

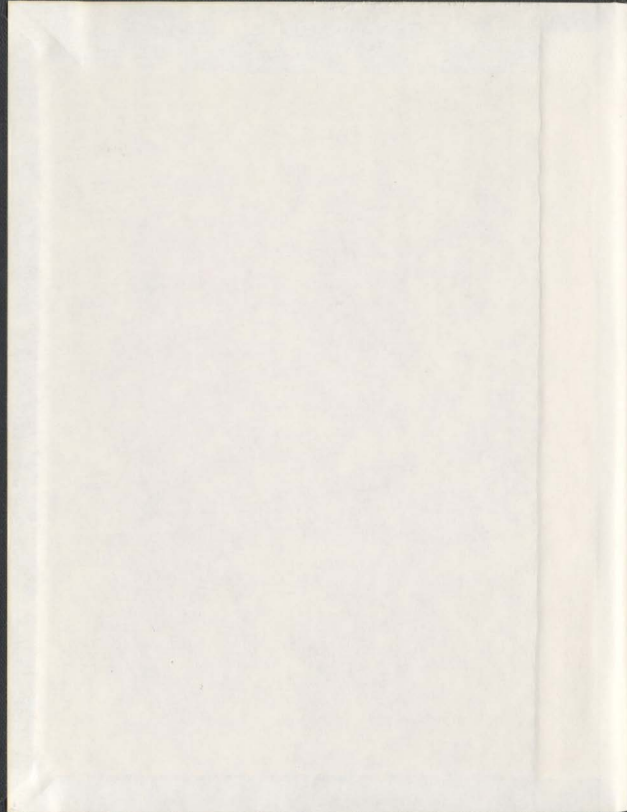
ENZYMES OF GLUTAMINE
METABOLISM IN RAT:
SUBCELLULAR LOCALIZATION
OF GLUTAMINASES IN LIVER
AND KIDNEY CORTEX AND
CHARACTERIZATION OF
 γ -GLUTAMYL TRANSFERRING
ACTIVITIES IN RAT
KIDNEY CORTEX

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ENZYMES OF GLUTAMINE METABOLISM IN RAT:
SUBCELLULAR LOCALIZATION OF GLUTAMINASES IN LIVER AND KIDNEY
CORTEX AND CHARACTERIZATION OF γ -GLUTAMYL TRANSFERRING
ACTIVITIES IN RAT KIDNEY CORTEX

by



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A Thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
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St. John's

Newfoundland

To My
Late Parents

ABSTRACT

Urinary excretion of ammonia facilitates excretion of strong acids and serves to conserve body stores of sodium. This ammonia is provided mainly by the renal hydrolysis of glutamine catalyzed by the glutaminase isoenzymes. The subcellular localization of the isoenzymes of glutaminase has been studied in rat kidney cortex and liver. Differential centrifugation and sucrose density gradient techniques demonstrated a mitochondrial localization for phosphate-dependent glutaminase in both liver and kidney cortex. Fractionation of isolated mitochondria by digitonin and by sonication revealed that phosphate-dependent glutaminase is located in the mitochondrial matrix compartment, a finding in agreement with its demonstrable latency.

The highest specific activity of phosphate-independent glutaminase was found in the microsomal fraction of rat kidney cortex on differential centrifugation. This fraction was also enriched in NADPH-cytochrome c reductase (endoplasmic reticulum marker), 5'-nucleotidase (plasma membrane marker), alkaline phosphatase, γ -glutamyltranspeptidase and maltase (brush border markers). Continuous sucrose density gradient studies on microsomal fractions showed that phosphate-independent glutaminase was located in the brush border membranes of rat kidney cortex. This enzyme is truly membranous as it could not be removed by sonication, salt treatment or pH alterations.

Further studies on phosphate-independent glutaminase and γ -glutamyl transferring activities of rat kidney cortex showed that phosphate-independent glutaminase enzyme appears to be a single hydrolase which

(iii)

catalyzes the hydrolysis of glutamine, glutathione, γ -glutamylhydroxamate and γ -glutamyl-p-nitroanilide. The enzyme activity was stimulated in the presence of maleate and competition between the substrates was observed. Phosphate-independent glutaminase activity was not attributed to the glutamine synthetase and it also appeared to be different from γ -glutamyltransferase activity. Phosphate-independent glutaminase and γ -glutamyltranspeptidase activities were lost to identical extents by various heat treatments, were removed identically from brush border membranes by papain treatment and were co-purified on Sephadex G-100. Glutamine was shown to inhibit γ -glutamyltranspeptidase competitively with γ -glutamyl-p-nitroanilide. These studies suggests that phosphate-independent glutaminase and γ -glutamyltranspeptidase represent different catalytic actions of the same enzyme.

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ABBREVIATIONS

AMP	- adenosine 5'-phosphate
ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
CoA	- coenzyme A
DTT	- dithiothreitol
DNA	- deoxyribonucleic acid
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethylene glycol bis (β -aminoethyl ether) - N, N'-tetraacetic acid
HEPES	- N-2-hydroxyethylpiperazine - N'-2-ethanesulfonic acid
h	- hour
nm	- nanometer (millimicron)
min	- minute
MES	- 2 (N-morpholino) ethanesulfonic acid
MSO	- methionine sulfoximine
NAD	- nicotinamide - adenine dinucleotide
NADH	- nicotinamide - adenine dinucleotide, reduced
NADP	- nicotinamide - adenine dinucleotide phosphate
NADPH	- nicotinamide - adenine dinucleotide phosphate, reduced
PDG	- phosphate-dependent glutaminase
PIG	- phosphate-independent glutaminase
PEPCK	- phosphoenolpyruvate carboxykinase
s	- second
Tris	- tris (hydroxymethyl) amino methane
TCA	- trichloroacetic acid
V _{max}	- maximum velocity

CHAPTER I
INTRODUCTION

During normal metabolism a carnivore produces large quantities of acid as by-products of metabolism. The weaker carbonic acid is converted to carbon dioxide and excreted by the lungs, whereas the stronger non-volatile acids, particularly, sulfuric and phosphoric, must be excreted by the kidney. It is apparent that not much of these acids can be excreted in the free form within the confines of observed urinary pH. These acids are buffered in the body by bicarbonate and transported to the kidney as neutral sodium salts. Therefore, if they were excreted in the urine in the same form, with the concomitant loss of fixed cations, buffer stores of the body would be progressively depleted and acidosis would supervene. Instead, these anions and an equivalent number of protons are excreted in the urine either as titratable acid or as neutral ammonium salts. The fixed cations are restored to the body as bicarbonate salts.

In this function, by producing and excreting ammonia, kidney plays a major role in the regulation of acid-base balance of the body. Normal man, maintained on a mixed diet, produces each day 40 to 80 mEq of strong metabolic acid. Of this, some 30 to 50 mEq are excreted in combination with ammonia and 10 to 30 mEq are excreted as titratable acid. The urinary excretion of ammonia adapts to the acid-base status of the organism. For example, in the ketoacidosis of uncontrolled diabetes some 300 to 500 mEq of acid are excreted daily in combination with ammonia (1). Similarly, in acidosis caused by prolonged starvation, loss of base or ingestion of acid, the enhancement of renal ammonia production is a crucial factor in the maintenance of acid-base balance within limits compatible with life. Failure of this mechanism

results in a dangerously severe acidosis such as, for example, occurs in uremic stage of chronic renal disease.

The facilitation of acid excretion by ammonia is brought about as follows (2):

1. Ammonia in solution exists as the readily permeant free base (NH_3) and as the poorly permeant ammonium ion (NH_4^+) in the pH-dependent equilibrium: $\text{NH}_3 + \text{H}^+ \longleftrightarrow \text{NH}_4^+$. At pH 7.0 about 99% of the total ammonia exists as NH_4^+ . At the lower pH values normally found in the urine the proportion as NH_4^+ is even greater.
2. NH_3 , formed in the renal tubular cells, rapidly diffuses into the tubular fluid and is largely converted to NH_4^+ . The importance of this lies in the inability of renal tubular cells to maintain H^+ gradients between tubular fluid and blood of greater than 800/1 (1). Thus, the kidney cannot form urine of pH less than about 4.6. Excretion of NH_3 at the acidification sites serves to buffer the protons of tubular fluid, thus facilitating further acid excretion.
3. Ammonia excretion permits the urinary excretion of acids such as sulfuric, acetoacetic and β -hydroxybutyric in the form of ammonium salts. This also operates as an important part of a sodium conservation mechanism since urinary disposal of the anions of acids would otherwise require excretion of an equivalence of plasma cations.

SOURCE, SITE OF PRODUCTION AND SECRETION OF AMMONIA

Nash and Benedict (3) were the first to demonstrate that ammonia was formed by the kidneys from some precursor in the arterial blood and was rapidly excreted in high concentrations into urine. Then, until 1943, there was considerable speculation concerning the identity of the

4

substrate from which the kidney produced ammonia. Many workers at different times suggested blood urea (4-6), amino acids (7,8) and the amide groups of plasma protein (9) as precursors of renal ammonia. In 1943 Van Slyke et al. (10) demonstrated that plasma glutamine is the major precursor of ammonia produced by the kidney of the acidotic dog. They observed that sufficient glutamine was extracted from the blood perfusing the kidney to account for all of the ammonia added to renal venous blood and for two-thirds or more of that excreted in the urine. In alkalosis, both the renal extraction of glutamine and the renal production of ammonia was reduced.

Subsequent studies showed that the infusion of glutamine, asparagine and certain other amino acids in normal (11) and acidotic dog (7,12) increases the rate of ammonia excretion in the urine. Bliss (7) demonstrated that this increase in rate of ammonia excretion in urine was also observed after administration of both natural and unnatural (L and D) isomers of certain amino acids. Lotspeich and Pitts (12) and Kamin and Handler (11) further demonstrated that some amino acids increase ammonia excretion when their plasma levels were elevated and others did not. Similar results were also observed when a series of amino acids were infused directly into one renal artery of the acidotic dog (13). On the basis of these findings amino acids are usually grouped with respect to their potency to increase ammonia excretion. In general, the amino acids fall into three groups of descending capacity in this regard: L-glutamine, L-asparagine, L- and D-alanine and L-histidine are highly effective; L-aspartic acid, glycine, L-leucine, L-methionine, and L-cysteine are moderately

effective; and L-glutamic acid, L-lysine and L-arginine are completely ineffective in stimulating ammonia excretion. Ammonia excretion following intravenous and renal infusion of an amino acid into an acidotic animal suggests the existence of the requisite enzymatic machinery for deaminating or deamidating that amino acid. It tells nothing at all about the contribution of that amino acid to the production of ammonia when its plasma concentration is within the normal range.

Shalhoub *et al.* (14) and Pitts *et al.* (15) using modern techniques to measure the concentrations of amino acids in arterial and renal venous plasma of acidotic dog showed that glutamine was extracted in far greater amounts than any other amino acid. This confirms the findings of Van Slyke *et al.* (10). Similar findings have also been reported in man (16) and extended to rats (17,18). Pitts and his coworkers (2) employing [^{15}N] glutamine showed that amide nitrogen of glutamine accounts for an average of 43.3% of the renal pool of ammonia whereas the amino nitrogen of glutamine accounts for 18.3%. Thus on an average, nearly two-thirds of the renal ammonia is derived from the two nitrogens of plasma glutamine.

Walker (19), using the micropuncture technique on amphibian kidneys, showed that ammonia was not detected in the glomerulus, proximal tubule and the first one-third of the distal tubular fluid. However, ammonia first appeared in samples collected from the middle of the distal tubule and increased in concentration as the fluid flows along the terminal parts of the nephron. The increase in concentration of ammonia exactly paralleled the increase in acidity of the urine (20).

However, studies on the rat showed that the glomerular filtrate was modestly acidified as it flows along the proximal tubule (21). Glabman *et al.* (22) were the first to demonstrate the contribution of proximal tubules to urine ammonia in rat kidney. Similar findings have been reported by Hayes *et al.* (23). In micropuncture studies on normal, acidotic and alkalotic rats, these authors observed that net ammonia secretion occurred in the proximal tubule and that all parts of the nephron added ammonia to the luminal fluid. In acidosis, the proximal tubule may contribute as much as 50 to 70% of the total urinary ammonia. A pattern of hydrogen ion secretion similar to that of ammonia secretion has also been found in rat kidney. Progressive acidification occurred in the proximal tubule (24,25), distal tubule and collecting ducts (21).

The results from stop-flow studies in the dog indicate that the site of ammonia secretion and urine acidification are coextensive in the distal portion of the nephron (26,27). In micropuncture studies on normal dogs, neither ammonia secretion (28) nor acidification (29) were observed in the proximal tubule; in contrast to the findings in normal rats. However, both became evident in the proximal tubule in chronic metabolic acidosis. The possibility of proximal tubular contribution to urine ammonia in the normal state cannot be discarded, for even if substantial, it could still be below the limit of sensitivity of the analytical procedure.

The plasma levels of glutamine have been shown either not to change appreciably in acute (30) or chronic (31) metabolic acidosis in dog or in chronic acidosis in rats (32) or to exhibit a modest

decrease in chronic acidosis in rats (18). Thus, it seems unlikely that glutamine supply is a controlling factor for the production of ammonia. Since transport of ammonia from cell to tubular fluid is an extremely rapid, passive process (33,34), it follows that the most likely sites of regulation of ammonia production are the transport of glutamine into cortical cells and its subsequent intracellular metabolism.

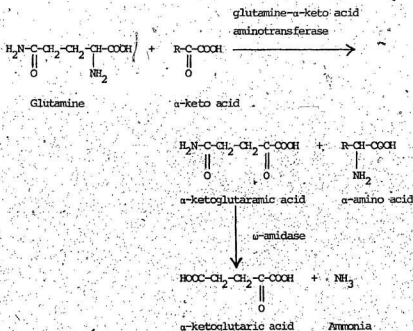
Glutamine can be provided to the cortical cells through both the luminal and anti-luminal surfaces (35,36). In fact, in chronic metabolic acidosis the fraction of plasma glutamine removed by the kidney is far higher than the fraction of plasma filtered so that glutamine transport through the anti-luminal surface occurs. Welbourne (37) suggested that the renal uptake of glutamine is not a concentrative process. Besides this, there is no data available to indicate how the transport of glutamine into cells is regulated.

However, regardless of the site and mechanism of glutamine uptake, that the subsequent metabolism of glutamine is under regulation is demonstrated by Hems and Brosnan (38) that the concentration of glutamine in renal cortex is decreased in both chronically and acutely acidotic rats. Similarly, a decrease in the concentration of glutamine was also reported in the various parts of the nephron of acidotic rat kidney (39). That increased ammonia excretion occurs in the face of decreased cellular concentrations of precursor argues for a facilitation of the enzymatic reactions leading to ammonia formation.

ENZYMES OF IMPORTANCE IN FORMATION OF AMMONIA

In 1935 Krebs (40) reported the enzymatic breakdown of glutamine and obtained evidence for the existence of different glutaminases in

α -ketoglutaramic acid, which is subsequently deamidated by an ω -amidase to give ammonia and α -ketoglutaric acid.¹



Errera (41) and Errera and Greenstein (42) demonstrated that glutaminase I is bound to insoluble particles of liver and kidney whereas, glutaminase II is located in the supernatant fraction. Shepherd and Kalnitsky (45) assigned glutaminase I to the "large granular fraction" of rat liver. Klingman and Handler (46) observed that pig kidney glutaminase was located exclusively in the mitochondria and that it was also stimulated by sulfate and arsenate ions. Lamar (47) reported that glutaminase I was found predominately in mitochondrial preparations and that another enzyme, "glutamohydroxamase", which

1. The glutaminase II activity was not investigated by me because of its reported localization in the soluble fraction (41,42) and because of its low activity (49).

is able to hydrolyze both glutamine and γ -glutamylhydroxamate was associated in the microsomal fraction. O'Donovan and Lotspeich. (48) studied the effects of different carboxylic acid on the rat kidney cortex mitochondrial glutaminase and observed that glutaminase is activated not only by phosphate and some other divalent inorganic anions, but also by a group of mono-di- and tricarboxylic acids, most of which are normal metabolites of Kreb's cycle.

Goldstein (49) suggested that glutaminase I is the predominant pathway for ammonia formation in kidney. This conclusion has been mainly drawn from his work which showed that the extractable activity of glutamine- α -keto acid aminotransferase in kidneys from normal and acidotic rats was much too low to account for the ammonia production observed in these states. Similarly, in dog kidney, the activity of glutaminase exceeds that of glutamine- α -keto acid aminotransferase by a factor of 20 (43).

Katunuma et al. (50,51) demonstrated the existence and separation of two different isoenzymes of glutaminase I in the kidney, liver and brain of rat. One isoenzyme which requires phosphate for maximal activity is usually referred to as phosphate-dependent glutaminase (PDG). The second isoenzyme which is not affected by phosphate and is highly activated by maleate has been referred to as phosphate-independent glutaminase (PIG). The two isoenzymes in rat kidney are found to have considerably different biochemical properties which are summarized in Table I.

Katunuma et al. (51) reported that both phosphate-dependent and

TABLE I
CHARACTERISTIC PROPERTIES OF THE GLUTAMINASE ISOENZYMES IN KIDNEY*

Properties	PDG	PIG
Activity without co-factor	-	+
Co-factor or activator	phosphate arsenate sulfate	maleate carbonate
K_m for glutamine	$4 \times 10^{-2} M$	$4 \times 10^{-3} M$
Optimum pH	8.2 - 8.7	7.2 - 7.6
Inhibition by PMS	strong	no effect
Inducibility (high protein diet)	+	-
Inhibition by glutamate	+	-

* From Katunuma *et al.* (51)

phosphate-independent glutaminase from the kidney, liver and brain of rat were recovered largely in the mitochondrial fraction. Franklin and Goldstein (52) studied the properties of renal glutaminase activity in adult, two-week old and newborn rats. They observed that the biochemical properties and intracellular location of phosphate-dependent glutaminase remained the same throughout development. Most of phosphate-dependent glutaminase activity was shown to be present in the mitochondrial fraction.

Experiments have been performed by many workers to establish the precise location of phosphate-dependent and phosphate-independent glutaminase within the mitochondria. Crompton *et al.* (53) reported that in pig kidney mitochondria, phosphate-dependent glutaminase was located in the mitochondrial matrix and that phosphate-independent glutaminase may be bound to the inner mitochondrial membrane. In another publication, Chappell *et al.* (54) assigned phosphate-independent glutaminase to the outer mitochondrial membrane. Kovačević (55) reported that in rat kidney phosphate-dependent glutaminase was found in the mitochondria whereas, phosphate-independent glutaminase was observed mainly in the microsomal fraction. He also suggested that phosphate-independent glutaminase could partially be associated with the outer mitochondrial membrane. The work reported in this thesis shows that phosphate-dependent glutaminase is located within the inner mitochondrial compartment whereas, phosphate-independent glutaminase was found in the brush border membranes of rat kidney cortex. Similar findings have also been observed in rat kidney by Curthoys and co-workers (56,57). Recently some workers (58,59) and my own studies

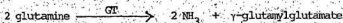
reported in this thesis suggest that phosphate-independent glutaminase is a catalytic function of γ -glutamyltranspeptidase.

A considerable body of information is available on phosphate-dependent glutaminase. The enzyme has been extensively purified from pig kidney and its properties have been studied (60). This enzyme can exist in three different forms each possessing different molecular weights, depending on the ionic environment. Although all forms of the enzyme exhibit kinetic differences they are all activated by phosphate and are inhibited by glutamate (61). Kvaume and Torgner (62,63) reported that purified phosphate-dependent glutaminase from pig kidney is activated by CoA and short chain acyl CoA derivatives and by low concentrations of long chain acyl CoA and inhibited by higher concentrations of long chain acyl CoA. Phosphate-dependent glutaminase has also been purified from rat kidney (64,65), which appears to be quite similar to the pig kidney enzyme in terms of its existence in different molecular aggregates.

It has long been known that metabolic acidosis results in an adaptive increase in phosphate-dependent glutaminase activity in the rat (66) but not in the dog (67). This increase in the rat is entirely due to an elevation of enzyme activity in the proximal convoluted tubules. Curthoys and Lowry (68) reported that phosphate-dependent glutaminase is principally found in distal straight and distal convoluted tubules in normal rats, but in acidotic animals there was a 20-fold increase in the activity in proximal convoluted tubules such that these structures now possessed the highest activity. Similar conclusions had earlier been arrived at by Karnovsky (69) using

histochemical techniques. Micropuncture studies in normal and acidotic rats have shown that the proximal tubule is responsible for at least 70% of excreted ammonia (22,23). Thus, it seems reasonable to propose that phosphate-dependent glutaminase in the proximal convoluted tubules is responsible for the production of the major part of the ammonia excreted.

Recently Welbourne (70) and Phenix and Welbourne (71) have postulated a new possibility for the production of renal ammonia from glutamine by the glutamyltransferase reaction. It is pointed out that glutamyltransferase activity found in kidney homogenates (glutamine + hydroxylamine \xrightarrow{GT} NH_3 + γ -glutamylhydroxamate) may also catalyze the following reaction:



Such a reaction for ammonia production would yield a ratio of one for ammonia production to glutamine utilization as compared to glutaminase I pathway (glutamine $\longrightarrow 2 \text{ NH}_4^+ + \alpha\text{-ketoglutarate}^{2-}$) where the ratio for ammonia production to glutamine utilization would be two.

Welbourne (32) has shown in experiments with perfused kidneys that a ratio of one for ammonia production to glutamine utilization was achieved with kidneys from both the normal and acidotic rats when high concentrations of glutamine (4 mM) was employed in the perfusate. At substrate concentration, approaching *in vivo* values (1 mM), in the perfusate resulted in a ratio of 1.38 with normal kidneys, but rose to 1.74 when acidotic kidneys were perfused. Thus, in addition to the fact that acidotic kidneys utilize glutamine more rapidly than normal

kidneys, these experiments clearly indicate a significant difference in the mechanism by which perfused acidotic and normal kidneys metabolize near physiological concentrations of glutamine. Phenix and Welbourne (71) explained this difference by suggesting that glutamine metabolism in normal kidneys predominately occurs via the glutamyltransferase reaction and in acidotic kidneys via the mitochondrial phosphate-dependent glutaminase (glutaminase I pathway). Evidence for this proposition came from the observations that acetazolamide (which inhibits glutamyltransferase activity) also inhibited ammonia production by normal kidneys, but had a much lesser effect in acidotic kidneys and from the finding of large quantities of presumed γ -glutamylglutamate in kidneys after perfusion with glutamine. Similar conclusions were also reached after using DL-methionine-DL sulfoximine (MSO) as an inhibitor of glutamyltransferase activity (32).

The major unsolved problem with Welbourne's postulate concerns with the fate of the γ -glutamyl peptides generated. They could not be allowed to accumulate *in vivo* as they do in the perfused kidney and a suitable description of their subsequent disposition is essential before the postulated role of glutamyltransferase in ammonia production is clarified. Secondly, the suggested glutamyltransferase reaction for ammonia production is not economically advantageous since for every mole of ammonia produced one mole of glutamine is utilized.

Welbourne's reported glutamyltransferase is shown to be capable of hydrolyzing both the L- and D-isomers of glutamine equally well. This enzyme seems to be different than the glutamyltransferase studied by Herzfeld (72) where the enzyme was capable of using only L-isomers

of substrate. However, Welbourne's postulated glutamyltransferase appears to be similar to the γ -glutamyltranspeptidase at least with regards to substrate specificity (73). Recently, Wadoux and Welbourne (74) showed that addition of MSO to the glutamyltransferase assay system reduced both ammonia production and glutamyltransferase activity. However, addition of MSO to the γ -glutamyltranspeptidase assay system had no effect on ammonia production and γ -glutamyltranspeptidase activity. It was suggested that γ -glutamyltranspeptidase does not significantly contribute to ammonia production and that it is the glutamyltransferase which appears to be the predominant source of ammonia production in the normal rat kidney. It may be possible that there are different glutamyltransferases in rat kidney.

Despite the uncertainty of the mechanism for ammonia production in non-acidotic kidneys there is no doubt that mitochondrial phosphate-dependent glutaminase is primarily responsible for the renal production of ammonia in acidosis.

THE MECHANISM OF ADAPTIVE CHANGES IN RATE OF AMMONIA EXCRETION

It has been well established in man and certain other mammals that the kidneys show a remarkable capacity to increase their rate of ammonia excretion in acidosis (75-78). Further, Pitts, (78) showed that in normal and acidotic dog ammonia excretion was inversely related to urine pH. At any given urine pH, the rate of ammonia excretion was higher in the chronic acidotic animal than in the normal animal. These observations are the basis for the view of "adaptation" of ammonia excretion in chronic metabolic acidosis. Since it has been conclusively shown that the rate of secretion of ammonia into acid urine is not limited by

diffusion across the cell membrane but rather by rate of ammonia production within the cell, changes in the production mechanism have been proposed to explain the adaptive increase observed during metabolic acidosis.

Davies and Yudkin (66) advanced the hypothesis that ammonia production in rats was regulated, at least in part, by changes in concentrations of relevant enzymes. They observed an increase in ammonia production from glutamine by renal cortical slices from acidotic rats and suggested that this was facilitated by an increased glutaminase activity. This postulate has been repeatedly confirmed in the rat (44; 76) and the guinea pig (79).

Rector *et al.* (76) reported a concordant relationship between glutaminase activity in homogenates of rat kidney and the increase in ammonia excretion during successive days of ammonium chloride administration. They showed that the rise in ammonia excretion preceded the rise in glutaminase activity, showing a slight increase during the first 24 hours but thereafter roughly paralleled the rise in glutaminase activity. There is, however, much evidence against the hypothesis that changes in renal glutaminase concentration are a necessary condition for changes in the rate of ammonia production and excretion in the intact rat. Leonard and Orloff (80) showed a four-fold increase in the rate of excretion of ammonia by the acidotic rats after only 24 hours of ammonium chloride administration without an appreciable rise in glutaminase activity. Recently Curthoys and Lowry (68) showed that the increase in phosphate-dependent glutaminase in acidosis was limited almost completely to the proximal convoluted tubules. It is, therefore,

possible that when this enzyme activity is measured in whole kidney, an increase in proximal tubular activity may not be detected because of the large activity in the distal tubule. Pollack *et al.* (81) and Rector and Orloff (67), however, found no adaptive increase in glutaminase activity of chronically acidotic dogs despite a marked increase in ammonia excretion. Thus, in the acidotic dog an adaptive increase in glutaminase activity is not required for the increased ammonia excretion. The best evidence against the hypothesis comes from the work of Goldstein (82) who showed that the induction of glutaminase I activity was prevented in early acidosis in rats with actinomycin D without affecting the increase in ammonia excretion. These findings have been confirmed by Bignall *et al.* (83). Although these authors stated that actinomycin D blocked both enzyme and urinary ammonia increase in acidotic rats, their results showed that rats given both actinomycin D and ammonium chloride excreted ten times as much ammonia as control rats although the activity of glutaminase I was identical in the two groups of rats. Animals given ammonium chloride excreted only slightly more ammonia than those given both ammonium chloride and actinomycin D. However, there was an increase in renal activity of glutaminase in animals given only ammonium chloride. From these studies it has been concluded that renal ammonia production in rat is not closely regulated by the extractable activity of glutaminase I. Thus, it has been proposed that the functional activity of glutaminase *in vivo* is increased as a consequence of some alteration of the intra-cellular milieu. This reasoning has led to the proposal that changes in renal ammonia production in rats are mediated primarily by activation or inhibition of existing glutaminase I. Indeed, it has been demonstrated that glutamate,

a product of the glutaminase I reaction, is an inhibitor of the activity of this enzyme in rat (84,85), pig (46), and dog (86), and has been suggested to be the mechanism by which the physiological regulation of ammonia excretion is achieved. Goldstein (85) suggested that the stimulation of renal gluconeogenesis during acidosis might contribute to the rise in renal ammonia excretion by lowering the concentration of glutamate. It was proposed that the renal concentration of glutamate could be regulated by acidosis and alkalosis (85). In acidosis the renal content of glutamate decreases and it has been suggested that this decrease de/inhibits glutaminase I. Whereas, in alkalosis the renal content of glutamate increases and this increases the inhibition of glutaminase I. Since glutaminase I is an intramitochondrial enzyme its activity should presumably be inversely related to the concentration of glutamate within the mitochondria. Measurements of intramitochondrial glutamate concentration are crucial to test the hypothesis. At present such measurements cannot be performed *in vivo*.

The content of glutamate in normal rat kidney is high enough to produce 55% inhibition of glutaminase I activity *in vitro*. In chronic acidosis there is approximately a 35% decrease in the renal content of glutamate (85). This decrease in glutamate would be expected to produce a 20% increase in glutaminase activity, resulting in an increase of ammonia production of comparable magnitude. The enhancement expected by this mechanism is still below that actually seen in chronic acidosis. Thus, if the behaviour of glutaminase I *in vivo* is similar to that observed by Goldstein (85) *in vitro* and if the measured total kidney contents of glutamate reflect the mitochondrial matrix concentration,

other factors would have to be involved to account for the magnitude of the increase in ammonia excretion in metabolic acidosis.

The redox state of nicotinamide adenine nucleotide in the mitochondria of the renal tubular cell is viewed as an important factor in renal ammoniogenesis. Preuss (87,88) correlated alterations in glutamate metabolism and ratios of oxidized to reduced pyridine nucleotide in the rat kidney. He observed a decrease in the content of glutamate, a rise in tissue ammonia and a rise in the ratio of both NAD/NADH and NADP/NADPH in response to chronic acidosis. The effects on the pyridine nucleotide ratios were accomplished through a significant rise in the concentration of NAD and NADP and a significant decrease in the renal concentration of NADPH. The concentration of NADH did decrease in acidosis, but not to a significant degree. He suggested that such changes in the redox state of pyridine nucleotide may play a role in lowering renal glutamate content through their effect on the glutamate dehydrogenase reaction and subsequently increase renal ammoniogenesis. Since glutamate dehydrogenase is an intramitochondrial enzyme; it is important to know the ratio of free NAD to NADH in mitochondria whereas, the author's studies on total tissue content of pyridine nucleotides do not clarify the distribution of the nucleotides between various cell compartments and also fail to differentiate between free and bound nucleotides.

Damian and Pitts (89), using pulse labeling technique, measured the rate of glutaminase I and glutamine synthetase reaction in rat kidney. The difference between these two rates would approximate the net rate of production of glutamate and ammonia or the net rate of

combination of glutamate and ammonia to form glutamine. They observed that the rate of glutaminase I reaction was increased in acidosis and decreased in alkalosis whereas the glutamine synthetase reaction was reduced in acidosis and was unchanged in alkalosis. Accordingly, in acidosis ammonia was produced in net amounts and excreted in the urine whereas in alkalosis glutamine was produced in net amounts and added to renal venous blood. It appears that in acidosis increased production of ammonia is a consequence not only of an increase in the glutaminase I reaction, but also of a decrease in the glutamine synthetase reaction. They suggested that these two reactions constitute an operationally reversible system, the net direction of which is influenced by the acid-base state of animal and by the magnitude of the renal pool of glutamine and α -ketoglutarate. These studies were performed on the basis of assumptions that kidney is a closed system, consisting of a single compartment, and that precursor is only converted into product. The kidney is obviously not a single compartment system. Instead, it consists of vascular, interstitial, luminal and several non-homogeneous cellular fluid compartments among which labeled precursors could be distributed at unknown rates.

Hird and Marginson (90) presented results which implicate oxidative processes in the production of ammonia from glutamine by rat kidney mitochondria and characterize glutamine as a rapidly oxidizable substrate for these mitochondria. They suggested that a permeability factor could be involved in the formation of ammonia from glutamine. Unfortunately, there is at present no definitive study on the transport of glutamine into rat kidney mitochondria or on the regulation of its hydrolysis in such mitochondria.

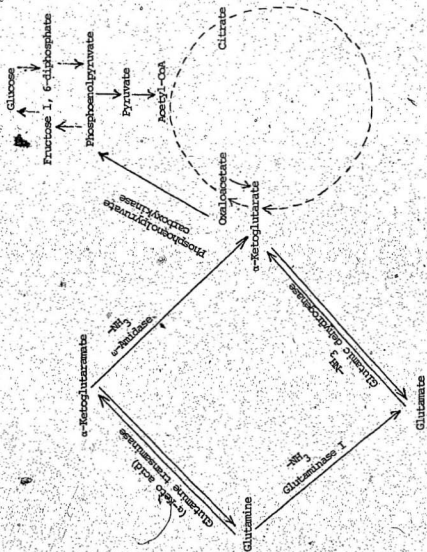
O'Donovan and coworkers (48,91,92) proposed that glutamine hydrolysis is regulated by the entry of activators into the mitochondrial matrix. They observed that the rate of mitochondrial swelling and ammonia production were both increased in acidosis and suggested that acidosis alters the structure of the mitochondrial membrane, making the enzyme more amenable to activation by certain compounds. However, it appears likely that the mitochondrial preparations used in their studies were not intact. Further, most of the experiments were carried out at very high concentrations of glutamine and activators. Therefore, these experiments are not relevant to the *in vivo* situation. Kovačević *et al.* (93) suggested that glutaminase activity in mitochondria may be "energy dependent". Thus, they observed that rapid production of ammonia required the presence of an oxidizable substrate. They also demonstrated an accumulation of glutamate inside the mitochondria during oxidation of glutamine. These data lead to the hypothesis that a prime factor in the regulation of glutaminase activity in mitochondria may lie in the disposal of the protons produced in the matrix. It was postulated that because of low permeability of kidney mitochondrial membrane to glutamate this amino acid accumulates inside the mitochondria and this leads to the inhibition of the enzyme by competition with phosphate. Crompton and Chappell (94) suggested that the entry of glutamine into pig kidney cortex mitochondria occurred in strict exchange for glutamate. This hypothesis was based on the poor swelling of these mitochondria in solution of glutamine and on finding of a constant (glutamine + glutamate) sum in the matrix. This mechanism has not yet been subjected to a rigid test in pig kidney mitochondria, but other studies tend to exclude it as an operative mechanism in rat kidney mitochondria (95).

Simpson and Sheppard (96) showed that conversion of $[U - ^{14}C]$ glutamine to $^{14}CO_2$ in dog kidney mitochondria was accelerated in media with a decrease in either pH or HCO_3^- . These data draw attention to the possibility of the regulation of mitochondrial glutaminase by intracellular alterations that are likely to occur in acidosis. Unfortunately, in these studies ammonia production was not measured. It need not necessarily parallel CO_2 production. Adam and Simpson (97) suggested that transport of glutamine across the mitochondrial inner membrane may be a rate limiting factor in ammonia production. In their studies, there was production and accumulation of $[^{14}C]$ glutamate in the mitochondrial matrix from $[^{14}C]$ glutamine but no glutamine could be found in the mitochondrial matrix even when higher concentrations of glutamine were employed. It was suggested that glutamine crosses the mitochondrial inner membrane by a carrier mediated step and it is deamidated immediately upon entry. Further, they also observed a four-fold increase in the radioactivity from $[^{14}C]$ glutamine in mitochondrial matrix from acidotic rats relative to normal rats. It was, therefore, proposed that this represents a stimulation of the transport mechanism in acidosis. However, an accumulation of glutamate could also be caused by a decreased exit of glutamate from the mitochondria. This is a real possibility since it has also been shown that uptake of $[^{14}C]$ glutamate is greatly depressed in mitochondria from acidotic rats and hence there may be a lower activity of the glutamate transport mechanism.

Recently Welbourne (98) studied the effect of adrenalectomy and adrenocorticoid administration on glutamine utilization. He observed that isolated perfused kidney from adrenalectomized rat had low rates

of ammonia production from glutamine and that this rate was markedly stimulated by the addition of small quantities of triamcinolone. This was brought about by facilitating the glutaminase I pathway. He suggested that the most likely site of action of glucocorticoid is on the mitochondrial membrane, increasing permeability to glutamine transport. This hypothesis needs to be investigated at the level of mitochondria before the site of action for glucocorticoid in this situation could be decided.

The mechanism whereby the kidney increases ammonia formation in response to acidosis is still unclear. The proposed hypothesis of Goodman et al. (99) that the enhanced gluconeogenesis in the kidney induced by acidosis is the primary driving force for the adaptive increase in ammonia production has lately received much attention. Goodman et al. (99) observed that gluconeogenesis from glutamine, glutamic acid, α -ketoglutarate and oxaloacetate is greater in slices from acidotic rats than from alkalotic rats. Glucose production from fructose or glycerol was unaltered in acidosis. For gluconeogenesis from glutamine, α -ketoglutarate is converted to oxaloacetate and then via a step catalyzed by phosphoenolpyruvate carboxykinase (PEPCK) to phosphoenolpyruvate and ultimately to glucose. The proposed relationship of gluconeogenesis to ammonia production is illustrated in Scheme 1. It was suggested that acidosis activates PEPCK activity. Thus, a fall in the concentration of α -ketoglutarate consequent to accelerated gluconeogenesis will pull the glutamic dehydrogenase step to the right, decrease tissue glutamate and accelerate glutamine deamidation. This hypothesis received experimental support when Alleyne (100), Bens and Brosnan (101), and Alleyne and Scullard (102), on the basis of analysis



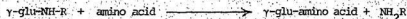
SCHEME 1. Relation Between Renal Gluconeogenesis from Glutamine
and Renal Production of Ammonia. [From Goodman *et al.*
(99)].

of the contents of metabolic intermediates in frozen kidney and direct estimates of PEPCK activity noted increased PEPCK activity in the kidney of acidotic rats. The increase in enzyme activity correlated with ammonia excretion in the intact animal. Both the PEPCK activity and ammonia excretion was shown to rise within 6 hours of induction of ammonium chloride acidosis without a change in glutaminase I activity. Further, Alleyne (103) showed that both gluconeogenesis and ammonia production were increased as part of early response to metabolic acidosis, but these processes were not as closely related as in the more chronically acidotic rat. Recently Alleyne and Roobol (104) found an increase in ammonia and glucose formation from glutamine *in vitro* by preincubating rat renal cortical slices in plasma isolated from acutely acidotic rats and suggested that a non-dialysable factor present in plasma of acutely acidotic rats may be responsible for the early increase in the urinary ammonia.

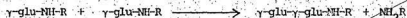
γ -GLUTAMYLTRANSFER ENZYME ACTIVITIES IN RAT KIDNEY CORTEX

Besides the well characterized mitochondrial phosphate-dependent glutaminase, there are a variety of enzymes which could catalyze the transfer of a γ -glutamyl moiety to various acceptors in rat kidney. γ -glutamyltranspeptidase catalyzes the transfer of the γ -glutamyl residue from glutathione and other γ -glutamyl compounds to various acceptors by the following reactions:

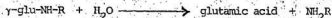
Transpeptidation:



Autotranspeptidation:



Hydrolysis:



γ -glutamyltranspeptidase is widely distributed in animal tissues, being highly active in kidney (105). It has been demonstrated both histochemically and biochemically that γ -glutamyltranspeptidase activity in the kidney is mainly concentrated in the brush border membranes of proximal tubules (106-109). This enzyme has been partially purified from hog, beef and rat kidney and some of its properties have been examined (110-112). The hydrolytic reaction of γ -glutamyltranspeptidase resembles the reaction catalyzed by phosphate-independent glutaminase. The work reported in this thesis demonstrates that phosphate-independent glutaminase is also localized in the brush border membranes of rat kidney cortex. This raises the possibility that both the phosphate-independent glutaminase and γ -glutamyltranspeptidase reactions are catalyzed by the same enzyme. Another activity, glutamohydroxamase, which accomplishes the hydrolysis of γ -glutamylhydroxamate and of glutamine has been demonstrated to be enriched in the microsomal fraction of rat kidney (47). Glutamine synthetase is also present in rat kidney and, indeed, has been suggested by O'Donovan and Lotspeich (91) and by Goldstein (113) to be responsible for the phosphate-independent glutaminase activity. γ -glutamyltransferase activity (glutamine + hydroxylamine \longrightarrow γ -glutamylhydroxamate + ammonia) is also present and this activity has often been attributed to glutamine synthetase (114). Recently Herzfeld (72) demonstrated that glutamine synthetase and γ -glutamyltransferase activities in rat liver and other tissues are not inextricably associated and therefore, may not be

catalyzed exclusively by the same protein. Welbourne (70) has pointed out a role for a glutamyltransferring activity, distinct from phosphate-dependent glutaminase in the production of ammonia in rat kidney from glutamine especially in normal kidney.

STATEMENT FOR THE RESEARCH PROBLEM

The work reported in this thesis was initiated in 1972. At that time it was well established that the major source of ammonia production in dog (15), man (16) and rat (17) kidney was glutamine, derived from the blood, which is hydrolyzed by renal glutaminase (41,49). Katunuma *et al.* (50,51) demonstrated the existence and separation of two different isoenzymes of glutaminase in the kidney, liver and brain of rat. One isoenzyme which requires phosphate for maximal activity is usually referred to as phosphate-dependent glutaminase. The second isoenzyme which is not affected by phosphate and is highly activated by maleate is referred to as phosphate-independent glutaminase. The sub-cellular localization of these isoenzyme is uncertain. Katunuma *et al.* (51) reported that most of the activity of glutaminases is found in the mitochondrial fraction. Errera (41) and Errera and Greenstein (42) have shown that phosphate activated glutaminase is bound to insoluble particles of liver and kidney whereas other workers (45) assigned this enzyme to the "large granular fraction" of rat liver. Klingman and Handler (46) observed that pig kidney glutaminase was located exclusively in the mitochondria. However, none of these workers have undertaken a comprehensive subcellular fractionation of liver or kidney cortex.

To understand the precise pathway of gluconeogenesis and ammonia production from glutamine and its regulation, it is important to know the precise localization of both the phosphate-dependent and phosphate-independent glutaminase in rat liver and kidney cortex.

In view of the plethora of γ -glutamyltransferring activities

reported in rat kidney and their possible importance in glutamine metabolism, I also studied these reactions so as to establish their subcellular localization and whether any or all of the enzyme activities are related.

CHAPTER II
MATERIALS AND METHODS

ANIMALS

Male Sprague-Dawley rats, weighing approximately 150 to 200 g were used in these experiments. The animals had free access to food and water. Animals were obtained from the Medical School, Memorial University of Newfoundland, or from the Canadian Breeding Laboratories, St. Constant, Quebec.

CHEMICALS

L-glutamine, L-glutamic acid, α -ketoglutaric acid, cytochrome c, L- γ -glutamyl-p-nitroanilide, L-glutamic acid γ -monohydroxamate, p-nitrophenol, creatine phosphate, p-nitrophenylphosphate, glutathione (reduced) and phenolphthalein glucuronic acid were purchased from Sigma Chemical Company, St. Louis, Mo., NAD, NADH, NADP, NADPH, and ADP were obtained from Boehringer Mannheim GmbH. [1 - 14 C] tyramine-hydrobromide (specific activity 6.04 mCi/m mole) was purchased from New England Nuclear Corp., Boston, Mass. All other chemicals were of analytical grade and were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J., British Drug House (BDH) Chemicals Ltd., Poole, England or from Fisher Scientific Company, Fair Lawn, N. J.

ENZYMES

Glucose 6-phosphate dehydrogenase, hexokinase, papain (2 x crystallized), L-glutamate dehydrogenase (Type II, in 50% glycerol containing sodium phosphate buffer, pH 7.3) and creatine phosphokinase were obtained from Sigma Chemical Company, St. Louis, Mo.

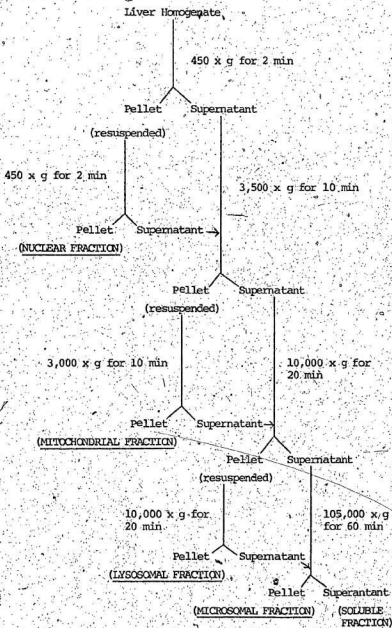
HOMOGENIZATION AND SUBCELLULAR FRACTIONATION OF LIVER

Animals were sacrificed by cervical dislocation and the liver

was immediately removed, weighed, chopped finely with a scissors and suspended in 9 times its weight of ice-cold 0.33 M sucrose containing 5 mM $MgCl_2$ and 2 mM HEPES, pH 7.4. The liver was then homogenized in a smooth-glass Potter-Elvehjem homogenizer at 470 rpm by 6 strokes with a loose-fitting teflon pestle (clearance 0.30 mm). After filtration through two layers of cheese cloth, the homogenate was fractionated by differential centrifugation into a nuclear fraction (N), a mitochondrial fraction (M), a lysosomal fraction (L), a microsomal fraction (P), and a soluble fraction (S), using essentially the method of de Duve *et al.* (115), as modified by Sedgwick and Hübscher (116). An outline of the procedure is shown in Scheme 2.

The homogenate was centrifuged in an International B-20 refrigerated centrifuge at $450 \times g$ for 2 min to give a sediment and a supernatant fraction. The supernatant was decanted, the sediment washed once by resuspension in homogenizing medium and centrifuged at $450 \times g$ for 2 min to give the nuclear (N) fraction. The supernatants were combined and centrifuged at $3,500 \times g$ for 10 min to give pellet and supernatant fractions. The pellet was resuspended and centrifuged at $3,000 \times g$ for 10 min to give a mitochondrial (M) fraction. The supernatants were combined and centrifuged at $10,000 \times g$ for 20 min to give pellet which was resuspended and centrifuged at $10,000 \times g$ for 20 min to give a lysosomal (L) fraction. The resulting supernatant was then centrifuged in a Beckman L-50 ultracentrifuge at $105,000 \times g$ for 60 min to sediment the microsomal (P) fraction. The final supernatant was termed the soluble (S) fraction. All the operations described above were performed at 0-4°C.

SCHEME 2. The Sub-Cellular Fractionation of Rat Liver
by Differential Centrifugation.



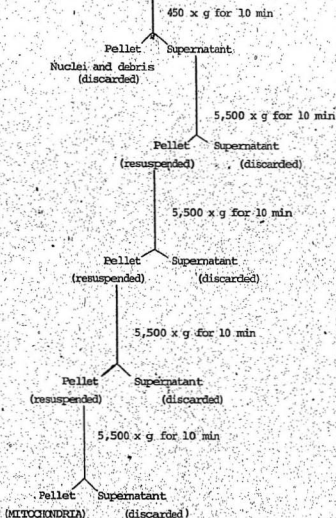
PREPARATION AND FRACTIONATION OF LIVER MITOCHONDRIA

Mitochondria were prepared from the liver by modifying the method of Chappell and Hansford (117), as shown in Scheme 3. The liver was chopped into small pieces and hand homogenized with a Potter-Elvehjem homogenizer (clearance 0.30 mm) in 0.25 M sucrose containing 1 mM EGTA and 3 mM Tris-HCl (isolation medium), pH 7.4. The homogenate was centrifuged at $450 \times g$ for 10 min to sediment nuclei and debris. The supernatant was then centrifuged at $5,500 \times g$ for 10 min to give a pellet (mitochondria) and supernatant fraction. The pellet was washed thrice by resuspending and recentrifugation to give mitochondria and was suspended in a small volume of isolation medium.

Mitochondria were diluted to a protein concentration of 20 mg per ml. The outer membrane was removed by the digitonin technique of Schnaitman and Greenawalt (118) with slight modifications. Aliquots of ice cold digitonin (10 mg per ml) were added to the suspension of mitochondria to give a final concentration of 1 mg of digitonin per 10 mg of mitochondrial protein. The resulting suspension was stirred gently for 15 min at 0° and then diluted with 2 volumes of isolation medium. The diluted suspension was centrifuged at $9,000 \times g$ for 10 min. The supernatant was removed and the pellet was washed once. The resulting pellet (inner membrane + matrix, IM + Ma) was resuspended in a small volume of isolation medium. The supernatants from the two centrifugations were pooled and centrifuged at $105,000 \times g$ for 60 min. The pellet (outer membrane, OM) was suspended in a small volume of isolation medium. The supernatant from this centrifugation was designated as the soluble (SOL) fraction.

SCHEME 3. Flow Chart for the Preparation of Mitochondria
From Liver.

Liver Homogenate
(Prepared by Hand Homogenization)



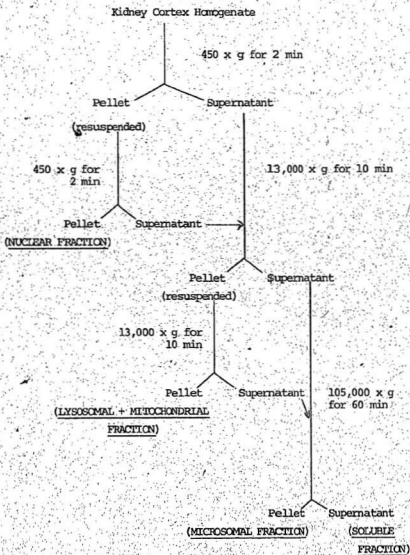
HOMOGENIZATION AND SUBCELLULAR FRACTIONATION OF KIDNEY CORTX

Animals were sacrificed by cervical dislocation and the kidneys were rapidly removed, demedullated and weighed. The cortices were then homogenized in a smooth glass Potter-Elvehjem homogenizer at 470 rpm by six strokes with a loose-fitting teflon pestle (clearance 0.30 mm). The homogenizing medium was 0.33 M sucrose containing 5 mM $MgCl_2$ and 2 mM HEPES, pH 7.4. The dilution was such that 1 g of kidney cortex was homogenized in 10 ml medium. After filtration through two layers of cheesecloth, the homogenate was fractionated by differential centrifugation. All operations were carried out at $0-4^{\circ}$.

The centrifugation procedures were essentially according to the method of de Duve *et al.* (115) with slight modifications as shown in Scheme 4. The homogenate was centrifuged in an International B-20 refrigerated centrifuge at $450 \times g$ for 2 min to give sediment and supernatant fractions. The supernatant was decanted, the sediment washed once by resuspension in homogenizing medium and centrifuged as above to give nuclear (N) fraction. The supernatants were combined and centrifuged at $13,000 \times g$ for 10 min to give lysosomal + mitochondrial (L + M) fraction which was also washed once. The resulting supernatant was then centrifuged in a Beckman L-50 ultracentrifuge at $105,000 \times g$ for 60 min to sediment the microsomal (P) fraction. The final supernatant was termed the soluble (S) fraction. Each sediment was resuspended in a small volume of homogenizing medium. These optimal homogenization and centrifugation conditions were chosen after carrying out a series of preliminary experiments. Experiments designed to yield individual lysosomal and mitochondrial fractions by differential

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SCHEME 4. The Sub-Cellular Fractionation of Rat Kidney
Cortex by Differential Centrifugation.



centrifugation were unsuccessful.

SUCROSE DENSITY GRADIENT ANALYSIS OF LYSOSOMAL + MITOCHONDRIAL FRACTION

For density gradient experiments, the combined lysosomal + mitochondrial fraction was prepared as described above and was washed three times. The resulting pellet was suspended in a small volume of 0.4 M sucrose containing 1 mM EDTA, pH 7.0. This fraction was layered either above a discontinuous sucrose gradient (prepared by layering 5 ml each of 1.9, 1.5, 1.4, 1.2, 1.1, 1.0, and 0.5 M sucrose containing 1 mM EDTA, pH 7.0 in each tube) or a continuous sucrose gradient (1.1 to 1.9 M sucrose containing 1 mM EDTA, pH 7.0) and was centrifuged in the SW 27 rotor of the Spinco L-50 ultracentrifuge at 25,000 rpm (82,500 \times g) for 2.5 h. After centrifugation, the contents of the tube were collected from the top in 1 ml aliquots using a ISCO model D-density gradient fractionator. All fractions were sonicated at 0° for 1 min (2 bursts for 30 s each), at 50 Watts output, with a Branson W 185 sonifier (micro probe) before enzyme assay.

PREPARATION AND FRACTIONATION OF KIDNEY CORTEX MITOCHONDRIA

Mitochondria were prepared from cortices by hand homogenization (8 strokes) with a Potter-Elvehjem homogenizer (clearance 0.30 mm) in 0.25 M sucrose containing 1 mM EGTA and 3 mM Tris-HCl (isolation medium), pH 7.4. These conditions were established after preliminary experiments in which different procedures were employed in order to obtain a preparation of intact mitochondria with least contamination with microsomes. The homogenate was centrifuged in an International B-20 centrifuge at 450 \times g for 10 min to sediment the nuclei and unbroken cells. The resulting supernatant was then centrifuged at 5,500 \times g for

10 min. The mitochondria was washed three times and were suspended in a small volume of isolation medium. These mitochondria were intact as evidenced by high respiratory control ratios, absolute latency of glutamate dehydrogenase, inability to oxidize external NADH, and the identical distribution of outer (rotenone-insensitive NADH-cytochrome c reductase) and inner (succinate-cytochrome c reductase) mitochondrial membrane markers after isopycnic centrifugation on a linear sucrose gradient.

Mitochondria were diluted to a protein concentration of 10 mg per ml and were fractionated either by the method of Schnaitman and Greenawalt (118) with slight modifications or by the digitonin-lubrol technique as described by Curthoys and Weiss (56). Since the method of Schnaitman and Greenawalt was established for rat liver mitochondria, it was necessary to find the optimal conditions for rat kidney cortex mitochondria. Therefore, in preliminary experiments, different concentrations of digitonin were used for the disruption of the outer membrane of mitochondria. Aliquots of ice cold digitonin were added to the suspension of washed mitochondria to give a final concentration of 2, 2.5 and 3 mg digitonin per 10 mg mitochondrial protein. The resulting suspension was stirred gently for 15 min at 0° and then diluted with 2 volumes of isolation medium. The diluted suspension was homogenized by hand and centrifuged at 9,000 x g for 10 min. The supernatant was removed and the pellet was washed once. The resulting pellet (inner membrane + matrix, IM + Ma) was resuspended in a small volume of isolation medium. The supernatants from the two centrifugations were pooled and centrifuged at 105,000 x g for 60 min. The pellet (outer membrane, OM)

was suspended in a small volume of isolation medium. The supernatant following removal of outer membrane was designated as the soluble (SOL) fraction.

When mitochondrial fractionation by the method of Curthoys and Weiss was performed, inner membrane + matrix particles prepared as above were treated as follows. To the inner membrane + matrix fraction was added $1/10^{\text{th}}$ volume of 0.1 M sodium borate - 1 M potassium phosphate - 1 M potassium pyrophosphate buffer, pH 8.9 and the suspension was allowed to incubate at room temperature for 10 to 20 min. An appropriate volume of a lubrol solution (20 mg per ml) was then added to a final concentration of 0.16 mg per mg protein. The suspension was allowed to incubate for 15 min at $0-4^{\circ}$ and was then diluted 2-fold with isolation medium containing 0.01 M sodium borate - 0.1 M potassium phosphate - 0.1 M potassium pyrophosphate, pH 8.9. The resulting solution was centrifuged at $105,000 \times g$ for 60 min to separate inner membrane (pellet) and matrix proteins (supernatant). The inner membrane pellet was suspended in a small volume of isolation medium.

ELECTRON MICROSCOPY

1 ml of different subcellular fractions obtained by differential centrifugation were fixed in the centrifuge tube with 1 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, containing 0.25 M sucrose. After waiting for 30 min the suspension were centrifuged. Electron microscopy of thin sections was performed, after post-fixation with osmium tetroxide and staining with uranyl acetate and lead citrate. A Philips FM 300 electron microscope was employed. These procedures were carried out by the electron microscopic division

of the Medical School.

DISRUPTION OF MITOCHONDRIA

In the experiments for the studies of intramitochondrial localization of glutaminase, the isolated mitochondria were separated into soluble and membranous fractions by the following treatments:

A. Sonication

Except where stated to the contrary the mitochondrial suspension was sonicated at 0° for 1 min (2bursts for 30 s each), at 50 Watts output, with a Branson W 185 D sonifier using a previously cooled microprobe. The suspension was then centrifuged at $105,000 \times g$ for 60 min into pellet and soluble fractions. The resulting pellet (membrane fraction) was resuspended in isolation medium.

B. Lubrol Treatment

Except where stated to the contrary aliquots of ice cold lubrol (20 mg per ml) was added to the suspension of washed mitochondria to a final concentration of 2 mg lubrol per 10 mg mitochondrial protein. The suspension was stirred gently at 0° for 15 min and was then centrifuged at $105,000 \times g$ for 60 min. The resulting pellet (membrane fraction) was resuspended in isolation medium.

C. Digitonin Treatment

Aliquots of ice cold digitonin (50 mg per ml)

was added to the suspension of washed mitochondria to a final concentration of 5 mg digitonin per 10 mg mitochondrial protein. The suspension was stirred gently at 0° for 15 min and was then centrifuged at $105,000 \times g$ for 60 min. The resulting pellet (membrane fraction) was resuspended in isolation medium.

SUCROSE DENSITY GRADIENT ANALYSIS OF THE MICROSOMAL FRACTION

For density gradient experiments, the microsomal fraction was prepared by differential centrifugation as described above and was washed once. Enzyme marker studies on such preparations showed less than 1% contamination with mitochondria and lysosomes. This fraction was layered above a continuous sucrose gradient (ranging from 1.1 to 1.9 M sucrose containing 1 mM EDTA, pH 7.0) and was centrifuged to equilibrium at 0-4° in the SW 27 rotor of the Spinco L-50 ultracentrifuge at 25,000 rpm ($82,500 \times g$) for 16 h. After centrifugation, the contents of the tube were collected from the top in 1 ml aliquots using an ISCO model D-density gradient fractionator.

DISRUPTION OF THE MICROSOMAL FRACTION

In experiments designed to remove the phosphate-independent glutaminase from the membranes of the microsomal fraction, these fractions were treated as follows:

A. Sonication

The microsomal fraction was sonicated at 0° for 2 min (four bursts for 30 s each), at 50 Watt

output, with a Branson W. 185 D sonifier using the microprobe.

B. KCl Treatment

The microsomal fraction (1.0 ml) was treated with 0.3 M KCl (1.0 ml) to give a final concentration of 0.15 M KCl in the solution. The suspension was allowed to stand at 0° for 30 min.

C. pH Alterations

The pH of the microsomal fraction was altered to either 5.5 or 8.5 by the addition of HCl or NaOH and the suspension was allowed to stand at 0° for 30 min.

D. NaCl Treatment

The microsomal fraction (1.0 ml) was treated with 0.3 M NaCl (1.0 ml) to give a final concentration of 0.15 M NaCl in the solution. The suspension was allowed to stand at 0° for 5 min.

E. EDTA Treatment

The microsomal fraction (1.0 ml) was treated with 0.108 M EDTA (1.0 ml), pH 7.0 to give a final concentration of 0.054 M EDTA in the solution. The suspension was allowed to stand at 0° for 5 min.

Combinations of the above treatments were also employed. The resulting suspensions were then centrifuged at 105,000 x g for 60 min.

The supernatant was removed and the pellet was resuspended in the isolation medium.

PAPAIN TREATMENT

In experiments designed to solubilize brush border enzymes, the washed microsomal fraction was treated with papain as described by George and Kenny (109). The incubation mixture contained (16 ml volume, pH 6.8), the microsomal fraction (about 10 mg. protein), 0.3 M sucrose, 12.5 mM KCl, 75 mM Tris-HCl buffer, 0.1 mM dithiothreitol, 0.1 mM cystein, and 0.9 mg. of papain. The suspension was incubated at 37° and at different time intervals samples (4 ml volume) were withdrawn, immediately cooled to 0° and centrifuged at 105,000 x g for 60 min. The supernatant was removed and the pellet was resuspended in isolation medium.

COLUMN CHROMATOGRAPHY

Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Montreal, Quebec) was swollen in 20 mM Tris-HCl buffer, pH 7.4 for 12 h at 60°. After cooling, the swollen gel was packed in a 30 cm x 1.5 cm column and equilibrated with the eluting buffer, containing 40 mM KCl, 1 mM EDTA, 0.5 mM DTT and 20 mM Tris-HCl, pH 8.0 at 4°. The microsomal fraction after treatment at 37° for 60 min with papain was centrifuged at 105,000 x g for 60 min. The supernatant (2.0 ml) was applied to the column and was eluted with the eluting buffer in the cold at a flow rate of 0.5 ml per min and 1.5 ml fractions were collected using an LKB ultrarac fraction collector.

REGIONAL DISTRIBUTION OF ENZYMES IN RAT KIDNEY

The regional localization of the different enzyme activities

within the kidney was determined by the procedure of Waldman and Burch (119). In these experiments, a cone shaped segment was cut from the center of each kidney, with the cortex as the base and the papilla at the apex. The cone was then cut free hand into five slices (two from cortex, two from medulla and one papilla). Comparable slices from different cones were pooled and homogenized in 0.33 M sucrose containing 5 mM $MgCl_2$ and 2 mM HEPES, pH 7.4. An aliquot of these homogenates was used for the determinations of enzyme activities.

ENZYME ASSAYS

A. Glutamate Dehydrogenase

The enzyme activity was assayed according to Brdiczka et al. (120), by measuring spectrophotometrically (at room temperature) the oxidation of NADH at 340 nm. The reaction mixture in a total volume of 3 ml contained: 5 mM EDTA, 0.22 mM NADH, 3 mM α -ketoglutaric acid, 2 mM ADP, 5 μ M rotenone, 50 mM triethanolamine buffer, pH 7.8. The reaction was started by the addition of ammonium sulfate to give a final concentration of 0.33 M. The enzymatic rates were calculated by using an extinction coefficient for NADH at 340 nm of 6.22×10^3 litre $\text{mol}^{-1} \text{cm}^{-1}$ (121).

B. Lactate Dehydrogenase

The enzyme activity was assayed spectrophotometrically (at room temperature) by following the oxidation of NADH at 340 nm as described by Morrison

et al. (122). The assay mixture in a final volume of 2.1 ml contained: 100 mM Tris-HCl buffer, pH 7.4, 0.15 mM NADH and 20 mM nicotinamide. The reaction was started by the addition of sodium pyruvate (pH 7.4) to give a final concentration of 2 mM. The enzymatic rate was calculated by using an extinction coefficient for NADH at 340 nm of $6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \text{ cm}^{-1}$ (121).

C. *NADPH- and Rotenone Insensitive NADH - Cytochrome c Reductase*

The enzyme activities were measured spectrophotometrically (at room temperature) by following the reduction of cytochrome c at 550 nm as described by Sottocasa *et al.* (123). The assay mixture in a final volume of 3 ml contained: 0.1 mM oxidized cytochrome c, 0.3 mM KCN, 50 mM phosphate buffer, pH 7.5, and when indicated, 5 μM rotenone. The reaction was started by the addition of either NADH or NADPH to a final concentration of 0.1 mM. An extinction coefficient (reduced-oxidized) for cytochrome c of $18.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \text{ cm}^{-1}$ (124) was employed in the calculation of the reaction rates.

D. *Succinate-Cytochrome c Reductase*

The enzyme activity was measured spectrophotometrically (at room temperature), by following the reduction of cytochrome c at 550 nm as described by

Sottocasa et al. (123). The assay mixture in a final volume of 2.1 ml contained; 0.1 mM oxidized cytochrome c, 0.3 mM KCN and 50 mM phosphate buffer, pH 7.5. The reaction was started by the addition of sodium succinate to a final concentration of 3 mM. An extinction coefficient (reduced-oxidized) for cytochrome c of 18.5×10^3 litre . mol⁻¹ cm⁻¹ (124) was employed in the calculation of the reaction rate.

E. β -Glucuronidase

The enzyme activity was measured according to the method of Gianetto and de Duve (125). The reaction mixture in a total volume of 1 ml contained: 0.00125 M phenolphthalein glucuronide and 0.075 M acetate buffer, pH 5.2. The reaction was started by the addition of enzyme source. After incubation at 37° for 15 min, the reaction was terminated by adding 3 ml of a solution containing 0.133 M glycine, 0.067 M NaCl and 0.083 M Na₂CO₃, pH 10.7. The suspension was centrifuged and the absorbance of the supernatant was measured at 560 nm. The concentration of phenolphthalein was determined from a calibration curve prepared from a sample of the pure substance.

F. Adenylate Kinase

The enzyme was assayed spectrophotometrically (at room temperature) by a modification of the method of Schnaitman and Greenawalt (118). The reaction

rate was measured at 340 nm by following the conversion of ADP to ATP + AMP and coupling the formation of ATP to the reduction of NADP with hexokinase and glucose-6-phosphate dehydrogenase. The assay mixture in a final volume of 3 ml contained: 15 mM glucose, 0.75 mM NADP, 10 units of hexokinase, 0.4 units of glucose-6-phosphate dehydrogenase, 0.33 mM sodium sulfide, 3 mM ADP, 5 mM $MgCl_2$ and 60 mM Tris-HCl buffer, pH 8.0. In this assay mixture 0.33 mM sodium sulfide replaced KCN as a cytochrome oxidase inhibitor. This substitution was made because the formation of a cyanide-pyridine nucleotide complex with an absorbance maximum at 320 nm could result in analytical errors. The assay mixture was allowed to incubate for about 5 min so as to permit the consumption of trace amounts of ATP present in the ADP. The reaction was started by the addition of enzyme source.

g. 5'-Nucleotidase

The enzyme activity was assayed as described by Pletsch and Coffey (126) in a final volume of 1 ml contained: 0.2 M KCl, 0.02 M disodium tartrate, 0.02 M $MgCl_2$, 0.1 M Tris-HCl buffer, pH 7.4. The reaction was started by the addition of AMP (pH 7.4) to give a final concentration of 0.01 M and allowed to proceed at 37° for 15 min. The reaction was terminated by the

addition of 0.2 ml of 30% trichloroacetic acid and the precipitated proteins were removed by centrifugation. The inorganic phosphate in the supernatant was measured by the method of Fiske and Subbarow (127).

II. Monoamine Oxidase

The enzyme activity was assayed according to Allmann et al. (128), by measuring the rate of conversion of [1 - 14 C] tyramine to [1 - 14 C] p-hydroxyphenylacetic acid and [1 - 14 C] p-hydroxyphenylacetaldehyde. The assay system in a volume of 1 ml contained: 0.3 mM [1 - 14 C] tyramine and 0.1 M phosphate buffer, pH 7.6. After incubation at 37°C for 15 min the reaction was terminated by the addition of 0.2 ml of 10 N HCl. The product was quantitatively removed by extraction of the assay mixture twice with 0.5 ml of ethyl acetate. The layer of ethyl acetate was washed with 1 ml of 0.1 N HCl and counted in a Beckman LS - 233 liquid scintillation counter using Aquasol as scintillation fluid. Control experiments were carried out to determine the amount of [1 - 14 C] tyramine extracted by ethyl acetate in absence of enzyme.

I. Phosphate-Dependent and Phosphate-Independent Glutaminase

These enzymes were routinely assayed by the procedure of Curthoys and Lowy (68). The initial incubation mixture for phosphate-dependent glutaminase in 1 ml

contained: 20 mM glutamine, 150 mM potassium phosphate (KH_2PO_4), 0.2 mM EDTA and 50 mM Tris adjusted to pH 8.6. The corresponding incubation mixture for phosphate-independent glutaminase in 1 ml contained: 10 mM glutamine, 60 mM maleate, 0.2 mM EDTA, adjusted to pH 6.6 unless otherwise indicated. These conditions are not optimal for the phosphate-independent glutaminase but were selected to minimize interference with the phosphate-dependent glutaminase. For either assay, the reaction was started by adding enzyme source. After incubation at 37° for 15 min the reaction was terminated by the addition of 0.05 ml of 10 N HCl. The resulting solution was centrifuged and an aliquot (0.2 ml) of the supernatant was used for the determination of glutamate. The glutamate was measured spectrophotometrically at 340 nm as described by Courthoys and Lowry (68).

While measuring the latency of phosphate-dependent glutaminase, the enzyme activity was also assayed at 340 nm by a continuous spectrophotometric procedure in which the production of ammonia was coupled to NADH oxidation. The assay mixture contains the following in 2 ml: 0.2 mM NADH, 20 mM α -ketoglutaric acid, 0.2 mM EDTA, 0.5 mM ADP, 0.25 M sucrose, 50 mM Tris-HCl, 75 mM phosphate, 5 μ M rotenone and 0.2 mg glutamic dehydrogenase. The

final pH was 8.2 and the reaction was started by the addition of glutamine to give a final concentration of 10 mM. Control experiments showed that NADH oxidation in the first 30 s was largely due to some preformed ammonia in the glutamine solution. Therefore, enzyme rates were measured after this time.

J. Glutamine Synthetase

The enzyme activity was assayed by the method of Herzfeld (72), based on the determination of the γ -glutamylhydroxamate formed when hydroxylamine replaces ammonia as a substrate, except that ATP regeneration was affected by creatine phosphate plus creatine phosphokinase as described by Vorhaben et al. (129). The reaction mixture (pH 7.3) in a total volume of 1 ml contained: 65 mM L-glutamate, 65 mM hydroxylamine, 16 mM $MgCl_2$, 6.4 mM ATP, 10 mM creatine phosphate, 15 units of creatine phosphokinase and 32 mM Tris-HCl buffer, pH 7.3. The reaction was started by addition of the enzyme source and the mixture was then incubated at 37° for 10 min. The reaction was stopped by the addition of 2 ml of ferric nitrate reagent [consisting of equal volumes of 40% (w/v) trichloroacetic acid and of 0.6 M ferric nitrate] as described by Iqbal and Ottaway (130). After rapid mixing and subsequent standing for 10 min the precipitated

proteins were removed by centrifugation and the absorbance of the supernatant was measured at 500 nm. The concentration of γ -glutamylhydroxamate was determined from a calibration curve prepared from a sample of the pure substance.

K. γ -Glutamyltransferase

The enzyme activity was assayed by the method of Thorndike and Reif-Lehrer (131) as modified by Herzfeld (72). The reaction mixture (pH 6.3) in a total volume of 1 ml contained: 260 mM glutamine, 40 mM hydroxylamine, 42 mM arsenate, 25 mM citrate, 0.2 mM ADP and 10 mM MnCl_2 . The reaction was started by the addition of enzyme and the mixture was then incubated at 37° for 10 min. The reaction was stopped by the addition of 2 ml of ferric nitrate reagent (130). After rapid mixing and subsequent standing for 10 min the precipitated proteins were removed by centrifugation and the absorbance of the supernatant was measured at 500 nm. The concentration of γ -glutamylhydroxamate was determined from a calibration curve prepared from a sample of the pure substance. A blank containing the total incubation mixture minus the enzyme was also used in each assay. The optical density of this blank was subtracted from the optical densities of the experimental mixtures.

L. γ -Glutamyltranspeptidase

The enzyme activity was assayed using γ -glutamyl-p-nitroanilide as substrate (73). The reaction mixture in a final volume of 1 ml contained: 5 mM γ -glutamyl-p-nitroanilide, 10 mM $MgCl_2$, 20 mM glycylglycine and 0.1 M Tris-HCl buffer, pH 8.2. The reaction was started with the enzyme and the mixture was then incubated at 37° for 3 to 5 min. The reaction was stopped by the addition of 2 ml of 1.5 N acetic acid and the absorbance of p-nitroaniline formed was measured at 410 nm against a reference solution containing the same components except that the enzyme was added after addition of acetic acid. The quantity of p-nitroaniline formed was determined from a calibration curve prepared from a sample of the pure substance.

M. Glutamohydroxamase

The enzyme activity was measured by a modification of the Lamar method (47). The incubation mixture in 1 ml contained: 5 mM γ -glutamylhydroxamate, 100 mM Na_2SO_4 and 20 mM HEPES, pH 7.6. After incubation at 37° for 15 min the reaction was terminated by the addition of 0.05 ml of 10 N HCl. The solution was centrifuged and an aliquot of the supernatant was used for the determination of glutamate as described by Curthoys and Lowry (68).

N. Maltase

The enzyme activity was measured by the method of Dahlqvist (132). The reaction mixture contained: 0.056 M maltose in 0.1 M sodium maleate buffer, pH 6.0 and enzyme source. After incubation at 37° for 60 min the reaction was terminated by the addition of 3 ml of Tris-glucose oxidase (TGO) reagent. The solution was mixed and let stand in a water bath at 37° for a further 60 min for the development of colour. The amount of glucose formed was measured at 420 nm against a reference solution containing the same components except that the substrate solution was added after the addition of TGO reagent. The TGO reagent contained: 100 ml of 0.5 M Tris-HCl buffer, pH 7.0, 125 mg glucose oxidase, 4 mg of peroxidase, 0.5 ml of 1% o-dianisidine and 1 ml of Triton X-100 (1:5 diluted with 95% ethanol).

O. Alkaline Phosphatase

The enzyme activity was measured using p-nitrophenylphosphate as substrate (133). The reaction mixture in a total volume of 1 ml contained: 0.0055 M p-nitrophenylphosphate and 0.05 M glycine buffer, pH 10.5. The reaction was started by the addition of enzyme source. After incubation at 37° for 10 to 15 min the reaction was terminated by adding 10 ml of 0.02 N NaOH. The mixture was centrifuged and the

absorbance of the supernatant was measured at 405 nm. The concentration of p-nitrophenol was determined from a calibration curve prepared from a sample of the pure substance.

DNA ESTIMATION

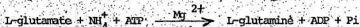
The DNA in subcellular fractions was extracted by a modification of the Schneider method (134). 0.5 ml aliquot of each fraction was mixed with 0.5 ml of ice cold 2.0 N perchloric acid. The suspension was centrifuged at $3,500 \times g$ for 10 min into pellet and supernatant. The pellet was washed twice with 0.7 N perchloric acid whereas, the supernatant was discarded. The washed pellet was finally suspended in 2 ml of 0.5 N perchloric acid and heated at 90° for 15 min. The suspension was then centrifuged at $3,500 \times g$ for 10 min. The pellet was discarded and an aliquot of the resulting supernatant was used for DNA measurement with diphenylamine reagent (135) using calf thymus DNA as standard.

PROTEIN ESTIMATION

Protein was measured either by the biuret method (136) following solubilization with deoxycholate (137), or occasionally by the Lowry method (138), using bovine serum albumin as standard.

CHAPTER III
LOCALIZATION OF PHOSPHATE-DEPENDENT GLUTAMINASE
IN RAT LIVER

The amino acid glutamine appears to be of major metabolic importance because of its involvement as a nitrogen source and as a constituent of most proteins. The amide and amino nitrogens of glutamine may be utilized in the biosynthesis of amino acids while the amide nitrogen may also be used in the synthesis of nucleotides, aminosugars and cofactors. In view of the metabolic significance of glutamine, it is not surprising to find that of all the plasma amino acids glutamine is present in the highest concentration. Glutamine is needed for the growth of several microorganism whereas it is not required in the diet of animals. Glutamine can be synthesized in animal tissues from glutamate and ammonia, by a reaction catalyzed by glutamine synthetase which is known to be present mainly in the microsomal fraction.



Because of the central role of glutamine in metabolism, it seems likely that glutamine synthetase must be subjected to regulatory mechanisms which could control the supply of glutamine available for different purposes. Glutaminase, glutamine α -keto acid transaminase and glutamine amidotransferase, are considered to be the most important enzymes involved in glutamine catabolism.

In mammals, glutamine is the most important intermediate in detoxification of free ammonia liberated from amino acids in various organs with the exception of liver and kidney. Glutamine which is produced in various organs is released into the blood and transported to the liver and kidney.

Glutamine transaminase catalyzes the reversible transfer of the α -amino group of glutamine to an α -keto acid to form the corresponding L-amino acid and α -ketoglutarate. The α -ketoglutarate is hydrolyzed to α -ketoglutarate and ammonia by α -amidase, an enzyme present in rat liver and in other tissues. The combined actions of glutamine transaminase and the α -amidase is usually referred to as glutaminase II. This enzyme system is present in the soluble portion of rat liver.

Glutamine is the major precursor for renal ammonia. During acidosis, the kidney extracts more glutamine from which more ammonia is produced (2). In spite of this increased renal catabolism of glutamine, the blood concentration of this amino acid remains unchanged in acidosis (30-32). This would infer that extrarenal production of glutamine has increased. Addae and Lotspeich (30) have shown that the increase in glutamine production occurs mainly in the liver in the dog. Since hepatic glutamine and renal ammonia production both increase in chronic acidosis, the question arises if there is any relationship between these two processes. Lund (139) showed that *in vivo* extrahepatic tissues can make a considerable contribution to the amount of circulating glutamine and concluded that rat liver is concerned with regulation of plasma glutamine concentrations and that the net production of glutamine is not a function of the liver under normal conditions. Further support for this view is provided by the results of Brosnan and Williamson (140) who found increase in alanine and aspartate amounts after administration of an ammonia load to rats *in vivo* with little or no increase in the amount of glutamine in livers from fed rats and a decrease in the amount in starved rats.

Lueck and Miller (141) studied the metabolism of glutamine in the isolated perfused rat liver under conditions simulating metabolic acidosis. It was suggested that metabolic acidosis suppresses both glutamine synthesis and glutamine utilization. However, the suppression of utilization was greater than that of synthesis so that the result was an apparent increase in net synthesis. Their studies supports the view that during acidosis *in vivo* the liver may contribute to the increased need of the organism for glutamine by effecting either a net output of glutamine as a result of a relative increase in synthesis or as a consequence of lessened uptake and catabolism of glutamine. To what extent the concentration of glutamine in the liver depends on the balance between glutamine synthetase activity and glutaminase activity is still uncertain.

Since inorganic ammonia is a direct substrate of carbamyl phosphate synthetase in the urea cycle, glutamine is not directly used for carbamyl phosphate synthesis. The amide nitrogen of glutamine which is transported to the liver should be hydrolyzed to inorganic ammonia prior to urea synthesis. Liver glutaminase is considered to play an important role in this metabolic pathway.

The localization of glutaminase in rat liver is not well established although Errera (41) has demonstrated that phosphate-dependent glutaminase is bound to insoluble liver particles and Shepherd and Kalnitsky (45) have assigned this enzyme to the "large granular" fraction.

It is, therefore, of importance to study the intracellular

localization of phosphate-dependent glutaminase to understand the precise subcellular pathway of gluconeogenesis from glutamine and also in understanding the control of the potential "futile cycle" that could be established by glutaminase and glutamine synthetase.

INTRACELLULAR DISTRIBUTION OF ENZYMES IN LIVER

Table II shows the distribution of phosphate-dependent glutaminase as compared to that of markers in different subcellular fractions isolated from a homogenate of rat liver. These results are presented in the manner proposed by de Duve et al. (115). The subcellular distribution patterns (Fig. 1) of typical nuclear (DNA), lysosomal (β -glucuronidase), mitochondrial (succinate-cytochrome c reductase), microsomal (NADPH-cytochrome c reductase) and cytoplasmic (lactate dehydrogenase) markers correspond to those observed by other workers (115,116,142). The intracellular distribution of phosphate-dependent glutaminase is very similar to that of succinate-cytochrome c reductase. Most of its activity is present in the mitochondrial fraction indicating that phosphate-dependent glutaminase is located in the mitochondria. The intracellular distribution of phosphate-independent glutaminase was not clear since there was not appreciable activity detected in any fraction under the conditions employed. It could well be that the conditions employed in my studies for the measurement of this enzyme activity are not satisfactory. Previous workers (143) reported that liver phosphate-independent glutaminase is extremely labile but they measured this enzyme activity in the presence of 10^{-3} M phosphate. The presence of phosphate in the assay system could well cause an artifact because phosphate is needed for phosphate-dependent glutaminase activity. Therefore, the

TABLE II

INTRACELLULAR DISTRIBUTION OF GLYCOGEN-DEPENDENT GLUTAMINE, SOME HYDROLYTIC ENZYMES AND OTHER CONSTITUENTS OF RAT LIVER

The enzymes, protein and DNA were assayed as described under "Materials and Methods". Protein and DNA are expressed in milligrams per g of liver (wet weight). The absolute values of enzyme activity in the total homogenate (H) are given in micromoles of substrate metabolized per g of liver (wet weight) per min. The results are given as means \pm S. D. Each value represents the average of at least four experiments. H is the total homogenate; N, the nuclear fraction; M, the mitochondrial fraction; L, the lysosomal fraction; P, the microsomal fraction and S, the final supernatant.

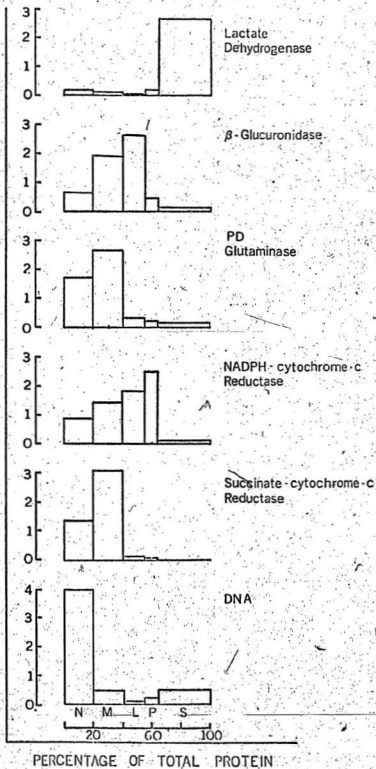
	Total Homogenate	Percentage of Recovery					Total
		H	N	M	L	P	
Protein	222.7 \pm 8.8	100	19.1 \pm 4.6	19.2 \pm 5.3	16.4 \pm 2.8	8.3 \pm 1.2	32.2 \pm 5.0
Deoxyribonucleic acid	1.9 \pm 0.13	100	72.6 \pm 7.3	9.3 \pm 3.9	2.0 \pm 0.33	1.9 \pm 0.26	18.7 \pm 6.9
Lactic dehydrogenase	443.8 \pm 25.7	100	3.0 \pm 0.94	1.4 \pm 0.28	0.4 \pm 0.10	1.5 \pm 0.45	84.4 \pm 5.1
NADPH-cytochrome c reductase	5.5 \pm 0.66	100	16.7 \pm 8.1	27.5 \pm 9.3	29.9 \pm 6.4	20.0 \pm 3.9	3.2 \pm 2.4
β -Glucuronidase	1.2 \pm 0.14	100	13.3 \pm 9.3	39.2 \pm 20.1	43.3 \pm 7.9	3.8 \pm 1.5	2.8 \pm 1.0
Succinate-cytochrome c reductase	4.2 \pm 0.23	100	25.9 \pm 7.1	61.0 \pm 8.1	1.2 \pm 0.38	0.2 \pm 0.08	88.5 \pm 6.2
Respirate-dependent glucosylase	1.4 \pm 0.31	100	30.5 \pm 9.1	49.3 \pm 8.2	4.6 \pm 1.1	2.2 \pm 0.83	5.0 \pm 2.2
							91.6 \pm 8.9

7

FIG. 1. Distribution pattern of phosphate-dependent glutaminase, DNA and some marker enzymes in fractions from rat liver. The relative specific activity of the fractions (percentage of total activity/percentage of total protein) are plotted on the ordinate. On the abscissa, the fractions are represented by their relative protein content, in the order in which they are isolated i.e., from left to right: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, soluble fraction. PD Glutaminase., Phosphate-dependent glutaminase.

11 N

RELATIVE SPECIFIC ACTIVITY



phosphate-independent glutaminase was not studied further in the liver. The presence of all the enzymes in the nuclear fraction is undoubtedly due in part to contamination with unbroken cells.

INTRAMITOCHONDRIAL LOCALIZATION OF PHOSPHATE-DEPENDENT GLUTAMINASE

Fig. 2 and Table III shows the distribution of phosphate-dependent glutaminase and of a number of mitochondrial marker enzymes in different subfractions of mitochondria obtained after treatment with digitonin. Rotenone-insensitive NADH-cytochrome c reductase was employed as a marker for the outer membrane, adenylate kinase for the inter-membrane space, glutamate dehydrogenase for the matrix and succinate-cytochrome c reductase for the inner membrane (118,123). Each of these mitochondrial enzymes is clearly associated with a specific isolated mitochondrial subfraction (Fig. 2). Glutaminase was distributed in the inner membrane + matrix (IM + Ma) fraction.

MATRIX LOCALIZATION FOR PHOSPHATE-DEPENDENT GLUTAMINASE

The results in Fig. 2 indicate that phosphate-dependent glutaminase is located either on the inner membrane or in the matrix of mitochondria. To distinguish between these possibilities the mitochondria were ruptured by a number of procedures and then centrifuged into membranous and soluble fractions. A higher concentration of digitonin was employed for complete disruption of mitochondria.

The distribution of glutaminase⁹ in these fractions was compared with that of glutamate dehydrogenase (matrix marker) and succinate-cytochrome c reductase (inner-membrane marker). Results are shown in Table IV. The recovery of all enzymes was greater than 85%. The

FIG. 2. Distribution pattern of phosphate-dependent glutaminase and some marker enzymes in subfractions isolated following digitonin treatment of liver mitochondria. The relative specific activity of the subfractions (percentage of total activity/percentage of total protein) are plotted on the ordinate. On the abscissa, the subfractions are represented by their relative protein content. IM + M₁, inner membrane + matrix fraction; OM, outer membrane; SOL, soluble fraction. PD Glutaminase., Phosphate-dependent glutaminase.

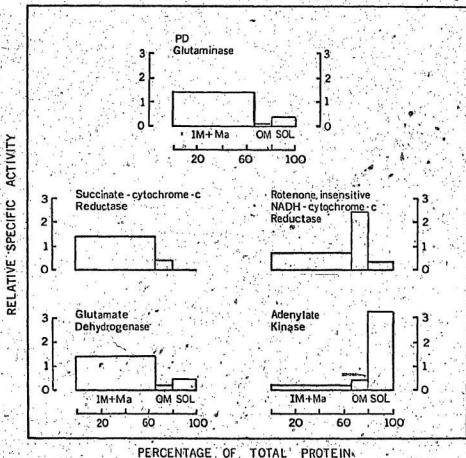


TABLE III

DISTRIBUTION OF PHOSPHATE-DEPENDENT GLUCOSE AND SOME MARKER ENZYMES IN RAT LIVER MITOCHONDRIA AFTER DIGITONIN FRACTIONATION

The enzymes and protein were assayed as described under "Materials and Methods". All specific activities are given in millimicroles per min per mg protein. The isolated mitochondria were treated for 15 min at 0° with digitonin (1 mg per 10 mg mitochondrial protein). The resulting suspension was fractionated as described under "Materials and Methods". The values given are from a typical experiment. Total activities are based on unfractionated mitochondria.

Sample	Protein	Glucose Dehydrogenase		Succinate-Cytochrome c Reductase		Rotenone-Insensitive NADH-Cytochrome c Reductase		Adenylate Kinase		Phosphate-Dependent Glucose-6-phosphate	
		Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity
Unfractionated mitochondria	100	162	100	210	100	185	100	238	100	62	100
Inner membrane + matrix	61.6	204	83.3	301	85.7	84	43.8	38	14.8	85	82.4
Outer membrane	12.5	34	2.4	68	5.4	290	32.1	20	4.1	18	3.6
Soluble	17.9	88	8.9	0	0	43	6.3	650	59.9	23	6.1
Recovery	92.0		94.6		91.1		82.2		79.8		92.1

TABLE IV

DISTRIBUTION OF PHOSPHATE-DEPENDENT GLUTAMINASE AND TWO MITOCHONDRIAL ENZYMES

BETWEEN SOLUBLE MITOCHONDRIAL PROTEIN AND MITOCHONDRIAL MEMBRANES

The enzymes were assayed as described under "Materials and Methods". The isolated mitochondria were either sonicated for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with lubrol (2 mg per 10 mg mitochondrial protein) or with digitonin (5 mg per 10 mg mitochondrial protein). The suspension was centrifuged at 105,000 x g for 60 min. The resulting pellet (membrane fraction) was resuspended before use. The values given are from a typical experiment.

Treatment	Fraction	Percentage Enzyme Activity		
		Succinate-cytochrome c reductase	Phosphate-dependent glutaminase	Glutamate dehydrogenase
Sonication	Membrane	100	76	65
	Soluble	0	24	35
Lubrol	Membrane	100	25	31
	Soluble	0	75	69
Digitonin	Membrane	94	28	25
	Soluble	6	72	75

phosphate-dependent glutaminase is not bound to the mitochondrial membranes and largely appears in the soluble fraction of mitochondria. The distribution of this enzyme is very similar to that of glutamate dehydrogenase. These results suggest that phosphate-dependent glutaminase is located in the matrix of liver mitochondria.

DISCUSSION

Glucose is readily formed from glutamine by the perfused liver (144). The presence of glutaminase in the mitochondrial matrix indicates that the path of gluconeogenesis from glutamine involves the amino acid entering the mitochondria and being deamidated there. The outer-membrane of mitochondria does not present a barrier to small molecules but the inner-membrane generally does. Therefore, the transport of glutamine across the inner-membrane may require a specific transport system. Additionally, a matrix localization for glutaminase implies that regulation of this enzyme will be effected by the concentration of metabolites in the mitochondrial matrix.

The presence in the same cell of enzymes carrying out opposing reactions poses special problems in metabolic regulation. Generally, a "futile cycle" can be prevented if the conditions that are favourable for one enzyme are inhibitory for the other and vice versa (145). The presence in liver cells of both glutaminase and glutamine synthetase would result in a net breakdown of ATP if they both proceeded simultaneously. In addition to direct modulation of enzyme activity, this potential "futile cycle" could be regulated by mitochondrial permeability. Glutamine synthetase is found on the surface of isolated microsomes and

it can be readily released by physiological concentrations of salt (146). Thus, the synthesis of glutamine is a cytoplasmic process. I have demonstrated glutaminase to be located in the mitochondrial matrix. Thus, for the "futile cycle" to operate glutamine must pass into the mitochondria and glutamate must exit. Little is known at present about glutamine movement across mitochondrial membranes, but it has been demonstrated that glutamate is transported by a specific transport system (147). Lueck and Miller (141) have clearly shown that the pH of the perfusion medium can determine whether the perfused liver utilizes glutamine or synthesizes it. It may well be that the control of glutamine and glutamate flux across the mitochondrial membranes is responsible for the *in vivo* regulation of the glutaminase-glutamine synthetase system.

The rat liver glutamine synthetase has been purified to apparent homogeneity and shown to be inhibited by alanine and glycine while α -ketoglutarate and citrate resulted in apparent activation of the enzyme (148). The reported findings that glutamine directly inhibits its own synthesis, and is more effective as an inhibitor when glutamate and ammonia are present in low concentration, suggests that intracellular glutamine levels may be important in the overall regulation of glutamine synthetase (149).

The liver glutamine synthetase is also strongly inhibited by phosphate (150) which may be of physiological importance in that glutaminase I is activated by phosphate. This would suggest that phosphate may offer reciprocal control of glutaminase and glutamine synthetase by activating the former and inhibiting the latter enzyme.

It is apparent that uncontrolled activity of glutaminase and glutamine synthetase represents a potentially wasteful metabolic situation, which must be normally prevented *in vivo* either by compartmentalization or by control mechanisms of some sort.



CHAPTER IV
LOCALIZATION OF GLUTAMINASE ISOENZYMES
IN RAT KIDNEY CORTEX



In the last chapter, it was demonstrated that liver phosphate-dependent glutaminase is located within the matrix compartment of mitochondria. Since the renal production and excretion of ammonia is an important factor in maintaining acid-base homeostasis both in normal animals and during metabolic acidosis, further studies were performed on the subcellular localization of both phosphate-dependent and phosphate-independent glutaminase in rat kidney cortex.

INTRACELLULAR DISTRIBUTION OF ENZYMES IN KIDNEY CORTX

Table V shows the distribution of phosphate-dependent and phosphate-independent glutaminases and that of markers in different fractions isolated from homogenates of rat kidney cortex. The results are shown graphically in Fig. 3 in the manner proposed by de Duve et al. (115). The subcellular distribution patterns (Fig. 3) of typical nuclear (DNA), lysosomal (β -glucuronidase), mitochondrial (succinate-cytochrome c reductase), endoplasmic reticulum (NADPH-cytochrome c reductase), plasma membrane (5'-nucleotidase) and cytoplasmic (lactate dehydrogenase) markers correspond to those observed by other investigators (151-153). Although the specific activity of NADPH-cytochrome c reductase is highest in the microsomal fraction, a significant amount of this enzyme is also present in the lysosomal + mitochondrial fraction. The amount of β -glucuronidase activity appearing in the soluble fraction can be explained by some disruption of lysosomes during the centrifugation procedure.

The intracellular distribution of phosphate-dependent glutaminase is very similar to that of succinate-cytochrome c reductase and

TABLE V

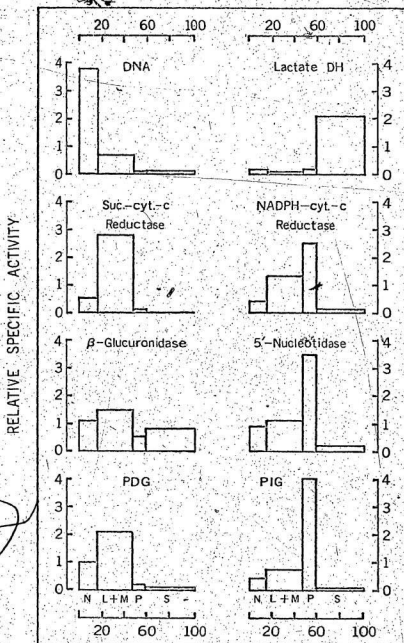
INTRACELLULAR DISTRIBUTION OF PROTEIN-DEPENDENT GLUCONIDASE, PROTEIN-DEPENDENT

GLUTAMINASE, SOME WATER ENZYMES AND CHEMICAL CONSTITUENTS OF RAT KIDNEY CORTEX

The enzymes, protein and DNA were assayed as described under "Materials and Methods". Protein and DNA are expressed in micrograms per g of kidney cortex (wet weight). The absolute values of enzyme activity in the total homogenate (H) are given in micrograms of substrate metabolized per g of kidney cortex (wet weight) per min. The results are given as means \pm S. D. Each value represents the average of at least four experiments. H is the total homogenate; N, the nuclear fraction; L + M, the lysosomal + microsomal fraction; P, the microsomal fraction; and S, the final supernatant.

	Total Homogenate	Percentage of Recovery					
		H	N	L + M	P	S	Total
Protein	179.0 \pm 4.2	100	15.5 \pm 5.0	32.2 \pm 4.2	10.4 \pm 1.6	45.6 \pm 5.8	103.7 \pm 5.3
Aspartic acid	7.7 \pm 0.1	100	11.8 \pm 1.0	6.0 \pm 0.32	0.85 \pm 0.03	92.6 \pm 5.2	97.9 \pm 7.0
Aspartate	92.2 \pm 0.39	100	7.3 \pm 7.2	44.4 \pm 2.9	25.6 \pm 6.5	5.8 \pm 7.0	81.1 \pm 10.5
NADH-cytochrome c reductase	21.4 \pm 3.4	100	15.3 \pm 3.5	66.3 \pm 9.6	2.1 \pm 0.91	3.2 \pm 3.0	86.9 \pm 8.6
Phosphate-dependent glucaminase	5.9 \pm 1.5	100	6.4 \pm 1.7	24.3 \pm 7.2	4.8 \pm 5.8	2.6 \pm 4.6	75.1 \pm 11.2
Phosphate-independent glucaminase	2.3 \pm 0.29	100	11.9 \pm 8.9	36.1 \pm 0.04	36.1 \pm 2.9	8.4 \pm 6.6	94.5 \pm 13.6
5-Hydroxylase	0.15 \pm 0.05	100	16.6 \pm 2.1	46.2 \pm 4.2	5.9 \pm 1.0	37.0 \pm 9.1	105.7 \pm 8.9
β -glucuronidase	7.51 \pm 0.23	100	8.5 \pm 1.0	88.8 \pm 4.6	0.85 \pm 0.06		98.2 \pm 3.8
Succinate-cytochrome c reductase							

FIG. 3.. Distribution pattern of phosphate-dependent glutaminase, phosphate-independent glutaminase, DNA and some marker enzymes in fractions from rat kidney cortex. The relative specific activities (percentage of total activity/percentage of total protein) of the fractions are plotted on the ordinate. On the abscissa, the fractions are represented by their relative protein content, in the order in which they are isolated, i.e., from left to right; N, nuclear fraction; L + M, lysosomal + mitochondrial fraction; P, microsomal fraction; S, soluble fraction. *Suc.-cyt.-c Reductase.*, succinate-cytochrome c reductase; *PDG.*, phosphate-dependent glutaminase; *Lactate DH.*, lactate dehydrogenase; *NADPH-cyt.-c Reductase.*, NADPH-cytochrome c reductase; *PIG.*, phosphate-independent glutaminase.



PERCENTAGE OF TOTAL PROTEIN

β -glucuronidase. Most of its activity is present in the lysosomal + mitochondrial fraction whereas the distribution of phosphate-independent glutaminase closely resembles that of NADPH-cytochrome c reductase and 5'-nucleotidase. The presence of these enzymes in the nuclear fraction is undoubtedly due in part to contamination with unbroken cells.

PURITY OF DIFFERENT SUBCELLULAR FRACTIONS OF KIDNEY CORTEX

The purity of different subcellular fractions was estimated by electron microscopy and enzymatic markers. Figs. 4, 5 and 6 show electron micrographs of the nuclear, lysosomal + mitochondrial and microsomal fractions respectively. In common with other studies of this type it is apparent that preparation of such fractions by differential centrifugation does not result in pure preparations of single organelles. Enzymatic assay of the fractions for different markers gives a quantitative measure of the extent of cross contamination.

MITOCHONDRIAL LOCALIZATION FOR PHOSPHATE-DEPENDENT GLUTAMINASE

As shown by differential centrifugation experiments (Fig. 3), the highest proportion of phosphate-dependent glutaminase is present in the lysosomal + mitochondrial fraction. The mitochondria in this fraction were, therefore, separated from the lysosomes by means of gradient centrifugation. Both discontinuous and continuous sucrose gradients were employed for the separation of lysosomes from mitochondria. Fig. 7 shows the result of a typical experiment in which the lysosomal + mitochondrial fraction (L + M) was layered above a discontinuous sucrose gradient and was centrifuged and fractionated as described under "Materials and Methods". The gradient profile (Fig. 7)

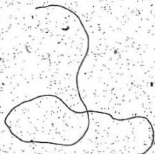


FIG. 4. Electron Micrograph of a Section of the Nuclear
Fraction Obtained by Differential Centrifugation.

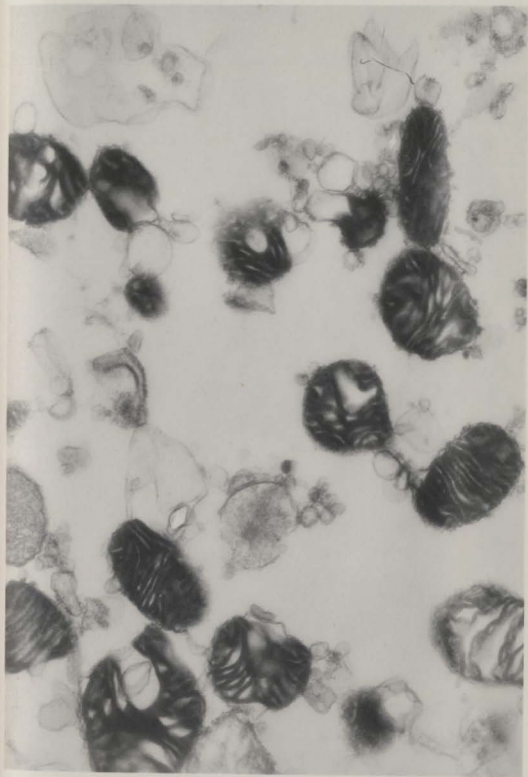
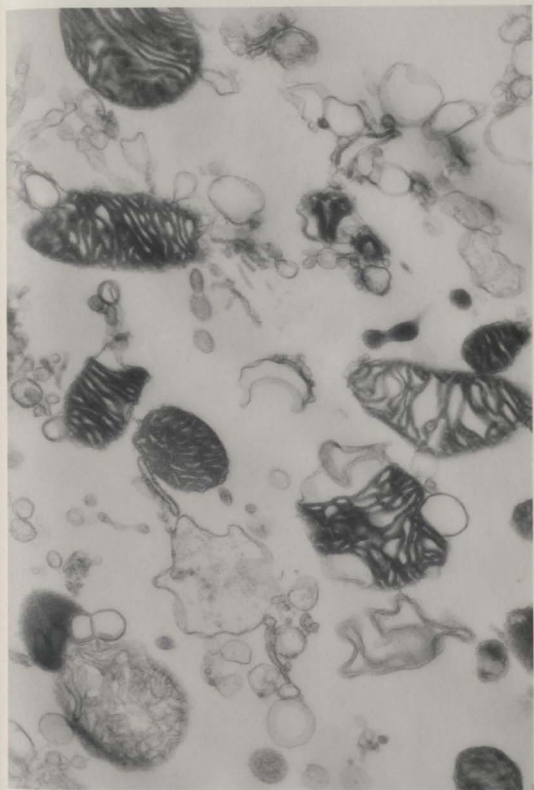


FIG. 5. Electron Micrograph of a Section of the Lysosomal
+ Mitochondrial Fraction Obtained by Differential
Centrifugation.




An electron micrograph showing a single, elongated, oval-shaped microsome with a textured internal structure. The microsome is positioned in the center-left of the frame. The background is light gray with scattered dark specks and a few faint, thin lines.

FIG. 6. Electron-Micrograph of a Section of the Microsomal Fraction Obtained by Differential Centrifugation.

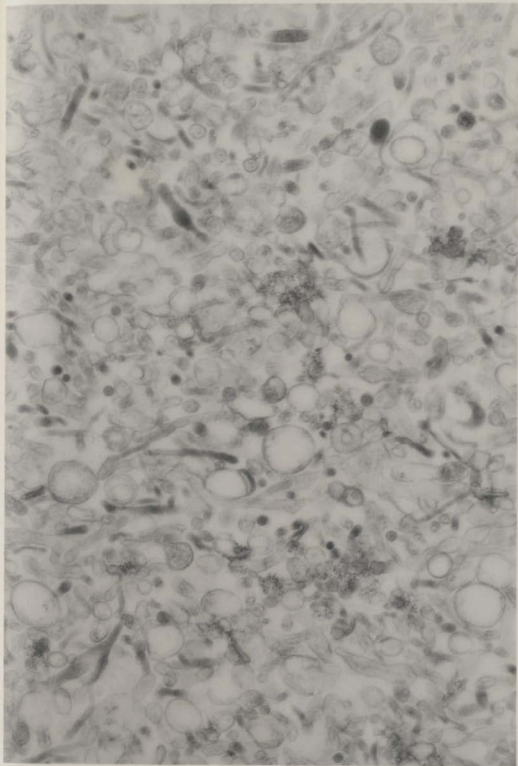
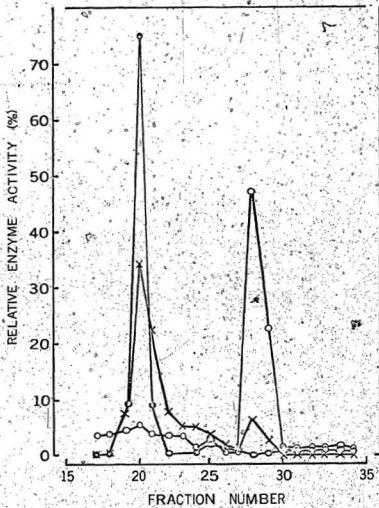


FIG. 7. Distribution pattern of phosphate-dependent glutaminase and marker enzymes after centrifugation in a discontinuous sucrose density gradient. The lysosomal + mitochondrial fraction was layered over the gradient and centrifuged for 2.5 h at 25,000 rpm as described under "Materials and Methods". After centrifugation the contents of each tube were collected in 1 ml aliquots which, on the *abscissa*, are represented in the order of their position in the tube, from meniscus (*left*) to bottom (*right*). The ordinate represents percentage of the relative enzyme activity recovered in each fraction. X—X, glutamate dehydrogenase; O—O, β -glucuronidase; ●—●, phosphate-dependent glutaminase.



shows clearly that the phosphate-dependent glutaminase is not contained in an organelle with β -glucuronidase activity. Phosphate-dependent glutaminase and glutamate dehydrogenase (mitochondrial marker) appeared in the same fractions. Since discontinuous gradients can artifactually sharpen bands at the interfaces of the different layers, experiments were also performed with a continuous sucrose gradient. Fig. 8 shows the result of a typical experiment in which the lysosomal + mitochondrial fraction (L + M) was layered above a continuous sucrose gradient and was centrifuged and fractionated as described under "Materials and Methods". Phosphate-dependent glutaminase and glutamate dehydrogenase (mitochondrial marker) appeared in the same fractions while β -glucuronidase was found in the denser fractions. The distribution of succinate-cytochrome c reductase (data not shown) was identical to that of glutamate dehydrogenase and phosphate-dependent glutaminase. This indicates that phosphate-dependent glutaminase is localized in the mitochondria.

LATENCY OF PHOSPHATE-DEPENDENT GLUTAMINASE

To test for latency, phosphate-dependent glutaminase was assayed in intact mitochondria and in mitochondria whose membranes were broken by various procedures. The enzyme activity was measured by both the continuous spectrophotometric method and by the method of Curthoys and Lowry, except that in the latter case the concentrations of glutamine and phosphate were 10 mM and 50 mM respectively, that 0.25 M sucrose was present to maintain isotonicity and that rotenone (2.5 μ M) was added to prevent oxidation of glutamate. Table VI shows the effect of sonication, lubrol treatment and digitonin treatment on the phosphate-dependent glutaminase activity. It is observed that these treatments

FIG. 8. Distribution pattern of phosphate-dependent glutaminase and marker enzymes after centrifugation in a continuous sucrose density gradient. The lysosomal + mitochondrial fraction was layered over the gradient and centrifuged for 2.5 h at 25,000 rpm as described under "Materials and Methods". After centrifugation the contents of each tube were collected in 1 ml aliquots which, on the *abscissa*, are represented in the order of their position in the tube, from meniscus (*left*) to bottom (*right*). The *ordinate* represents percentage of the relative enzyme activity recovered in each fraction. X-X, glutamate dehydrogenase; O-O, β -glucuronidase, ●-●, phosphate-dependent glutaminase.

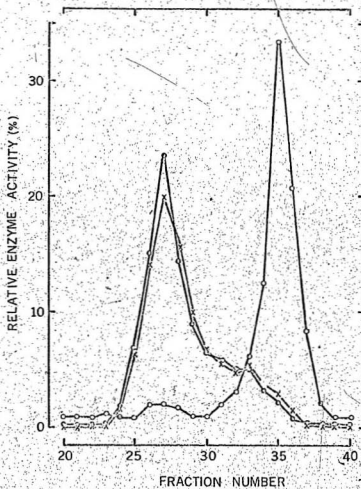


TABLE VI
 PHOSPHATE-DEPENDENT GLUTAMINASE IN KIDNEY CORTEX MITOCHONDRIA:
 EFFECT OF DIFFERENT TREATMENT
 ON PHOSPHATE-DEPENDENT GLUTAMINASE ACTIVITY

Mitochondria were either sonicated for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with lubrol (2 mg per 10 mg mitochondrial protein) or with digitonin (5 mg per 10 mg mitochondrial protein) before use. The values given are from a typical experiment.

Treatment	Percentage Enzyme Activity	
	Using continuous spectrophotometric method	Using Curthoys and Lowry method
None	100	100
Sonication	425	350
Lubrol	245	325
Digitonin	750	610

caused marked increases in enzyme activity irrespective to the assay method used for the measurement of activity. These results suggest that the enzyme is located within the mitochondrial permeability barrier (i.e., within the inner-membrane).

INTRAMITOCHONDRIAL LOCALIZATION OF PHOSPHATE-DEPENDENT GLUTAMINASE

In these studies, rotenone-insensitive NADH-cytochrome c reductase was used as a marker for the outer-membrane; adenylate kinase as a marker for the inter-membrane space; glutamate dehydrogenase for the matrix and succinate-cytochrome c reductase for the inner-membrane. These enzymes are commonly used as markers in liver mitochondria (118, 123). That their use as markers in kidney cortex mitochondria is valid was established by two types of experiments. In the first type of experiment the latency of these enzymes was determined. In the second type of experiment the localization (membrane or soluble fraction) of the enzymes was determined after disruption of the mitochondria. Results from such experiments are shown in Tables VII and VIII. The adenylate kinase was shown not to exhibit latency (Table VII) and was localized in the soluble fraction of the mitochondria after membrane rupture (Table VIII); therefore, it is in the inter-membrane space. Similarly, glutamate dehydrogenase was latent and soluble, therefore, it is localized in the matrix. Rotenone-insensitive NADH-cytochrome c reductase was not latent but was membranous, therefore, it resides in the outer-membrane. Succinate-cytochrome c reductase was both latent and membranous, therefore, it is located in the inner-membrane. Employing these marker enzymes to define the various mitochondrial compartments an independent confirmation of the internal localization

TABLE VII

LATENCY OF MARKER ENZYMES OF RAT KIDNEY CORTEX MITOCHONDRIA

The enzymes were assayed as described under "Materials and Methods" except that 0.25 M sucrose was present to maintain isotonicity. Mitochondria were either sonicated for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with lubrol (2 mg per 10 mg mitochondrial protein) or with digitonin (5 mg per 10 mg mitochondrial protein) before use. The values given are from a typical experiment.

Treatment	Percentage Enzyme Activity			
	Adenylate Kinase	Glutamate Dehydrogenase	Succinate-Cytochrome c Reductase	Rotenone Insensitive NADH-Cytochrome c Reductase
None	100	100	100	100
Sonication	94	380	290	93
Lubrol	98	450	460	96
Digitonin	100	660	740	97

TABLE VIII

DISTRIBUTION OF DIFFERENT ENZYMES BETWEEN THE SOLUBLE MITOCHONDRIAL FRACTION AND THE MITOCHONDRIAL MEMBRANE FRACTION

The enzymes were assayed as described under "Materials and Methods". 100% activities are based on unfractionated mitochondria. The isolated mitochondria were either solubilized for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with ligand (2 mg per 10 mg mitochondrial protein) or with digitonin (5 mg per 10 mg mitochondrial protein). The suspension was centrifuged at 105,000 x g for 60 min. The resulting pellet (membrane fraction) was resuspended before use. The values given are from a typical experiment.

Treatment	Fraction	Percentage Enzyme Activity			
		Adenylate Kinase	Glutamate Dehydrogenase	Succinate-cytochrome c Reductase	Potassium Insensitive NADH-cytochrome c Reductase
Solubilization	Unfractionated mitochondria	100	100	100	100
	Membrane Soluble	13	42	82	80
	Recovery	86	51	3	4
Ligand	Unfractionated mitochondria	100	100	100	100
	Membrane Soluble	14	20	88	87
	Recovery	74	64	0	0
Digitonin	Unfractionated mitochondria	100	100	100	100
	Membrane Soluble	11	15	86	89
	Recovery	80	79	0	0
		91	94	86	89

of phosphate-dependent glutaminase in mitochondria was demonstrated using the digitonin technique of Schnaitman and Greenawalt (118). In preliminary studies with kidney cortex mitochondria, 2.0, 2.5 and 3.0 mg digitonin per 10 mg mitochondrial protein were used to establish the best concentration of digitonin for the separation of outer and inner mitochondrial membranes. A concentration of 2 mg digitonin per 10 mg mitochondrial protein was found to be optimal for the separation of outer and inner membranes in kidney cortex mitochondria without release of matrix enzymes (Fig. 9). Higher concentration resulted in substantial release of glutamate dehydrogenase from the matrix. Therefore, this quantity (2 mg digitonin per 10 mg mitochondrial protein) of digitonin was employed in subsequent experiments. This is double the recommended ratio of digitonin to protein for rat liver mitochondria (118,154). However, the protein concentration (10 mg per ml) in these experiments was one-tenth of that used by Schnaitman et al. (118,154), so that the actual concentration of digitonin in solution was less in these experiments. It is, therefore, suggested that the digitonin concentration as well as the digitonin to protein ratio may be crucial for these experiments.

Table IX and Fig. 10 show the distribution of phosphate-dependent glutaminase and a number of mitochondrial marker enzymes in different subfractions of mitochondria obtained after treatment with digitonin. It is clear that phosphate-dependent glutaminase is distributed in the inner-membrane + matrix (IM + Ma) fraction. This confirms the internal localization of phosphate-dependent glutaminase indicated by latency experiments (Table VI).

FIG. 9. Distribution pattern of phosphate-dependent glutaminase and some marker enzymes in subfractions isolated following treatment of rat kidney cortex mitochondria with different concentrations of digitonin. The relative specific activity of the subfractions (percentage of total activity/percentage of total protein) are plotted on the ordinate. On the abscissa, the subfractions are represented by their relative protein content. IM + Ma, inner membrane + matrix fraction; OM, outer membrane; SOL, soluble fraction. RT, NADH-cyt.-c Reductase., rotenone insensitive NADH-cytochrome c reductase; Glutamate DH., glutamate dehydrogenase.

SERIES (A) - digitonin concentration 2.0 mg/10 mg mitochondrial protein.

SERIES (B) - digitonin concentration 2.5 mg/10 mg mitochondrial protein.

SERIES (C) - digitonin concentration 3.0 mg/10 mg mitochondrial protein.

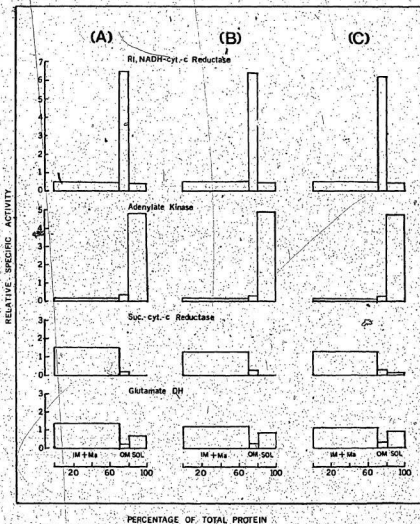


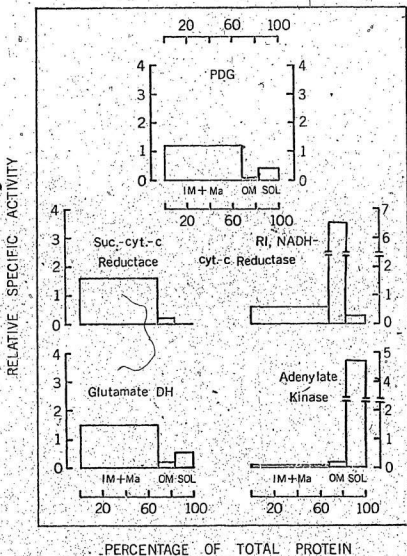
TABLE IX

DISTRIBUTION OF PHOSPHATE-DEPENDENT GLUCOSE AND SOME OTHER ENZYMES IN THE KIDNEY CORTEX MITOCHONDRIA AFTER DIGITONIN FRACTIONATION

The enzymes and protein were assayed as described under "Materials and Methods". All specific activities are given in millimoles per min per mg protein. The isolated mitochondria were treated for 15 min at 0° with digitonin (2 mg per 10 mg mitochondrial protein). The resulting suspension was fractionated as described under "Materials and Methods". The values given are from a typical experiment. Total activities are based on unfractionated mitochondria.

Sample	Protein	Glucose Dehydrogenase		Succinate-Cytochrome c Reductase		Ketone-Isomaltase		Adenylate Kinase	Phosphate-Dependent Glucosylase	
		(μ)	(μ)	(μ)	(μ)	(μ)	(μ)		(μ)	(μ)
Unfractionated mitochondria	100	368	100	216	100	109	100	227	100	484
Inner membrane + matrix	68.8	421	91.4	325	103.0	53	33.3	37	11.4	207
Outer membrane	7.2	85	1.9	535	11.1	706	46.6	75	2.4	42
Soluble	15.8	130	10.3	0	0	41	6.1	1,107	77.1	61
Recovery	91.8		103.6		104.1		86.0		90.9	84

FIG. 10. Distribution pattern of phosphate-dependent glutaminase and some marker enzymes in subfractions isolated following digitonin treatment of rat kidney cortex mitochondria. The relative specific activity of the subfractions (percentage of total activity/percentage of total protein) are plotted on the ordinate. On the abscissa, the subfractions are represented by their relative protein content. IM + M₂, inner membrane + matrix fraction; OM, outer membrane; SOL, soluble fraction. PDG., phosphate-dependent glutaminase; *sup.*, *cyt.-c* Reductase; succinate-cytochrome c reductase; Glutamate DH., glutamate dehydrogenase; RI, NADH-*cyt.-c* Reductase., rotenone insensitive NADH-cytochrome c reductase.



DISRUPTION OF MITOCHONDRIA

The above experiments clearly showed that phosphate-dependent glutaminase is latent and is distributed with the inner-membrane + matrix (IM + Ma) fraction. It was, therefore, important to disrupt the mitochondria by different treatments in order to achieve optimal conditions for the separation of mitochondria into soluble and membranous fraction and subsequently for the localization of phosphate-dependent glutaminase.

Table X shows the distribution of marker enzymes between soluble mitochondrial protein and mitochondrial membranes after different treatments. When the mitochondria are sonicated for varying periods, the fraction of glutamate dehydrogenase activity released in the soluble fraction is greater in those fractions sonicated for the longer times. It is clear that 1 min. sonication treatment results in more than 50% release of glutamate dehydrogenase in the soluble fraction. The longer (2 min) treatment results in the appearance of the membranous (succinate-cytochrome c reductase) enzyme activity in the soluble fraction. Therefore, in further experiments the disruption of mitochondrial membranes was achieved by sonication for 1 min. A final lubrol concentration of 2 mg per 10 mg mitochondrial protein results in a good release of soluble enzymes into soluble fraction. Similar results were obtained with a final concentration of 5 mg digitonin per 10 mg mitochondrial protein. Therefore, in subsequent experiments, a final concentration of 2 mg lubrol or 5 mg digitonin per 10 mg mitochondrial protein was used for the disruption of mitochondrial membranes.

TABLE X

DISTRIBUTION OF PHOSPHATES BETWEEN SOLUBLE MITOCHONDRIAL PHOSPHATES AFTER DIFFERENT TREATMENTS. The enzymes were assayed as described under "Materials and Methods". The isolated mitochondria were either sonicated for the indicated times, treated for 15 min at 0° with lubrol (1 or 2 mg per 10 mg mitochondrial protein) or with digitonin (3 or 5 mg per 10 mg mitochondrial protein). The suspension was centrifuged at 105,000 \times g for 60 min. The resulting pellet (membrane fraction) was resuspended before use. 100% activity was assigned to the unfractionated preparation. The values given are from a typical experiment.

Treatment	Percentage Enzyme Activity					
	Succinate-cytochrome c dehydrogenase			Glutamate dehydrogenase		
	Membrane	Soluble	Recovery (%)	Membrane	Soluble	Recovery (%)
Sonication	94	6	97	64	36	89
	91	8	95	39	61	92
	66	34	86	14	86	86
Lubrol	100	0	83	44	56	86
	100	0	81	27	73	90
Digitonin	100	0	83	42	58	87
	100	0	89	18	82	89

MATRIX LOCALIZATION FOR PHOSPHATE-DEPENDENT GLUTAMINASE

The latency experiments and those reported in Table IX and Fig. 10 indicate a location for the phosphate-dependent glutaminase either on the inside of the inner-membrane or in the matrix. To distinguish between these possibilities the mitochondria were ruptured by a number of procedures and then centrifuged into membranous and soluble fractions. The distribution of phosphate-dependent glutaminase in these fractions was compared with that of glutamate dehydrogenase (matrix marker) and succinate-cytochrome c reductase (inner-membrane marker). Results are shown in Table XI. The phosphate-dependent glutaminase is not bound to the mitochondrial membranes and largely appears in the soluble fraction of mitochondria. The distribution of this enzyme is similar to that of glutamate dehydrogenase.

When this work was reported, Curthoys and Weiss (56) suggested that phosphate-dependent glutaminase was contained in the inner mitochondrial membrane of rat kidney. In their studies they used a digitonin-lubrol technique for the disruption of mitochondria which was different from the techniques used for the experiments reported in this thesis. Therefore, mitochondria were fractionated into different subfractions by the digitonin-lubrol technique as described by Curthoys and Weiss (56) to establish the intramitochondrial localization of phosphate-dependent glutaminase. Table XII shows the distribution of phosphate-dependent glutaminase, succinate-cytochrome c reductase and glutamate dehydrogenase in different subfractions of mitochondria obtained after treatment with digitonin-lubrol. The highest specific activity of phosphate-dependent glutaminase was observed in the matrix subfraction of mitochondria but

TABLE XI

DISTRIBUTION OF PHOSPHATE-DEPENDENT GLUCOSE DEHYDROGENASE BETWEEN SOLUBLE MITOCHONDRIAL PROTEIN AND MITOCHONDRIAL MEMBRANES

The enzymes were assayed as described under "Materials and Methods". All specific activities are given in millimicroles per min per mg protein. Total activities are based on unfractionated mitochondria. The isolated mitochondria were either sonicated for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with lural (2 mg per 10 mg mitochondrial protein) or with digitonin (5 mg per 10 mg mitochondrial protein). The suspension was centrifuged at 105,000 x g for 60 min. The resulting pellet (membrane fraction) was resuspended before use. The values given are from a typical experiment.

Treatment	Fraction	Succinate-Cytochrome c Dehydrogenase		Phosphate-Dependent Glucose Dehydrogenase		Glutamate Dehydrogenase	
		Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity
Sonication	Unfractionated mitochondria	186	300	140	100	209	100
	Membrane Soluble	38	6	10	2	51	4
	Recovery	33	91	110	35	236	95
Lateral	Unfractionated mitochondria	162	100	107	100	154	100
	Membrane Soluble	280	85	54	25	70	23
	Recovery	0	0	142	67	185	61
Digitonin	Unfractionated mitochondria	156	100	128	100	219	100
	Membrane Soluble	38	82	44	28	88	13
	Recovery	0	0	168	78	308	80
			82		92		93

TABLE XII

DISTRIBUTION OF PHOSPHATE-DEPENDENT GLUCOSE AND TWO MITOCHONDRIAL ENZYMES

IN RAT KIDNEY CORTEX MITOCHONDRIA AFTER DIGITONIN-LIBREL FRACTIONATION

The enzymes and protein were assayed as described under "Materials and Methods". All specific activities are given in millimoles per min per mg protein. The isolated mitochondria were treated with digitonin-lubrol and were fractionated as described under "Materials and Methods". The values given are from a typical experiment.

Sample	Protein (μ)	Glucose-6-phosphatase		Succinate-Oxidase c		Phosphate-Dependent Glucosidase	
		Specific Activity	Total Activity (μ)	Specific Activity	Total Activity (μ)	Specific Activity	Total Activity (μ)
Unfractionated mitochondria	100	332	100	192	100	286	100
* Outer membrane	8.8	46	1.7	27	1.8	26	1.5
Soluble	12.3	76	7.6	0	0	54	6.9
Inner membrane	31.8	162	19.6	348	89.2	430	31.1
Nucleic	35.6	455	62.6	0	0	590	49.6
Recovery	90.5		91.3		91.0		89.1

enrichment (increased specific activity compared to unfractionated mitochondria) was also observed in the inner-membrane fraction. In these studies mitochondrial preparations from rat kidney cortex were used whereas, Curthoys and Weiss used whole kidney. Therefore, mitochondrial preparations from whole kidney were also used to establish the localization for phosphate-dependent glutaminase. Similar results were observed as reported in Table XII when whole rat kidney mitochondria were used for the studies.

WASHING OF LYSOSOMAL + MITOCHONDRIAL FRACTION IN ORDER TO REMOVE MICROSOMES

As shown in Fig. 3, a significant amount of NADPH-cytochrome c reductase was observed in the lysosomal + mitochondrial fraction. There was also a considerable activity of phosphate-independent glutaminase in the lysosomal + mitochondrial fraction. Therefore, the lysosomal + mitochondrial fraction was washed in order to see if phosphate-independent glutaminase and NADPH-cytochrome c reductase activity could be removed from lysosomal + mitochondrial fraction. Fig. 11A and 11B shows the distribution of different enzymes in thrice washed lysosomal + mitochondrial fraction. A small amount of NADPH-cytochrome c reductase and phosphate-independent glutaminase was observed in the washings (Fig. 11A). However, when the lysosomal + mitochondrial fraction was washed thrice by resuspending and recentrifuging at $5,500 \times g$ for 10 min, most of NADPH-cytochrome c reductase and phosphate-independent glutaminase activity was observed in the washings (Fig. 11B). Most of the phosphate-dependent glutaminase and glutamate dehydrogenase activity was observed in the washed lysosomal + mitochondrial fraction. These



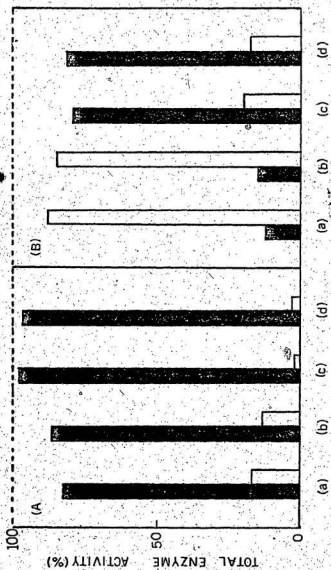
FIG. 11(A). Washing of rat kidney cortex lysosomal + mitochondrial fraction and the distribution of enzymes in thrice washed lysosomal + mitochondrial fraction , and in the washings . The activity of the unwashed lysosomal + mitochondrial fraction was calculated as 100%. The results given are the average of those obtained with four samples. (a), NADPH-cytochrome c reductase; (b), phosphate-independent glutaminase; (c), glutamate dehydrogenase; (d), phosphate-dependent glutaminase.

FIG. 11(B). As in Fig. 11(A) except that the washings were carried out by centrifugation at 5,500 x g.



results indicate that phosphate-independent glutaminase could be washed out from lysosomal + mitochondrial fraction and that the phosphate-independent glutaminase found in the lysosomal + mitochondrial fraction was due to contamination of this fraction by microsomes.

In these studies the intactness of mitochondria in lysosomal + mitochondrial fraction was also established in order to rule out the possibility that rupture and removal of outer mitochondrial membrane could result in an incorrect localization of phosphate-independent glutaminase. The intactness of mitochondrial outer-membrane in lysosomal + mitochondrial fraction was established by showing that succinate-cytochrome c reductase activity was completely latent when assayed in the lysosomal + mitochondrial fraction under isotonic conditions (Table XIII). Since cytochrome c cannot penetrate through the outer-membrane (155), such latency is evidence for the intactness of this membrane. The distribution of monoamine oxidase (outer-membrane marker) in different fractions (Table XIV) was very similar to that of succinate-cytochrome c reductase (Table V).

DISCUSSION

Although much work has been reported on the subcellular fractionation of rat liver based on the technique of de Duve et al. (115), there was no good differential centrifugation method, to my best of knowledge, for the subcellular fractionation of rat kidney cortex. Therefore, I developed a method for the fractionation of this tissue. The method employed for the preparation of kidney cortex subcellular fractions was based on that of de Duve et al. (115), but involved modifications of their procedure. Gentle homogenization with a loose pestle gave a

TABLE XIII
SUCCINATE-CYTOCHROME C REDUCTASE ACTIVITY IN THE LYSOSOMAL
+ MITOCHONDRIAL FRACTION OF RAT KIDNEY CORTEX

The enzyme activity was assayed as described under "Materials and Methods" except that 0.25 M sucrose was present to maintain isotonicity. The lysosomal + mitochondrial fraction was assayed for enzyme activity before and after lubrol treatment (2 mg/10 mg protein) at 0° for 15 min. The values given are from a typical experiment.

Treatment	Specific Activity (millimicromoles/min/mg protein)
None	0
Lubrol	92

TABLE XIV
INTRACELLULAR DISTRIBUTION OF MONAMINE OXIDASE OF RAT KIDNEY CORTEX

The enzyme was assayed as described under "Materials and Methods". The absolute value of enzyme activity in the total homogenate (H) is given in micromoles of substrate metabolized per g of kidney cortex (wet weight) per min. The results are given as mean \pm S. D. Each value represents the average of three experiments. H, is the total homogenate; N, the nuclear fraction; L + M, the lysosomal + mitochondrial fraction; P, the microsomal fraction and S, the final supernatant.

Enzyme	Total Homogenate	H	Percentage of Recovery				
			N	L + M	P	S	Total
Monamine oxidase	0.041 ± 0.004	100	13.8 ± 3.4	78.2 ± 4.4	1.9 ± 0.8	0	93.9 ± 2.9

separation of subcellular structures with a high degree of reproducibility and good recoveries of enzymatic activity. The purity of the individual subcellular fraction was tested by the use of marker enzymes regarded as specific for each subcellular component. The distribution and the relative specific activities of marker enzymes with respect to phosphate-dependent and phosphate-independent glutaminase in different subcellular fractions are shown in Table V and Fig. 3. The distribution pattern of different markers is comparable to those reported by other workers (151-153). The presence of NADPH-cytochrome c reductase in lysosomal + mitochondrial fraction appears to be due to the contamination of this fraction with microsomes since most of this enzyme activity could be washed out after washing of lysosomal + mitochondrial fraction (Fig. 11B). The results obtained with differential (Fig. 3) and gradient (Figs. 7,8) centrifugation showed that phosphate-dependent glutaminase is located in the mitochondria of rat kidney cortex. A similar conclusion has also been reported by other workers (52,55). Further studies on mitochondria (Tables IX, XI and XII) suggest that phosphate-dependent glutaminase may be located in mitochondrial matrix or loosely attached to the inside of inner mitochondrial membrane. Crompton et al. (53) reported that phosphate-dependent glutaminase in pig kidney is a soluble enzyme localized in the matrix compartment of the mitochondrion. Ourthoys and Weiss (56) suggested that phosphate-dependent glutaminase is associated with the inner-membrane of the rat kidney mitochondria. The results presented by these workers for enzyme activities are in terms of micromoles per min per ml of fraction and no final volumes and protein concentrations are reported. Hence, no specific activities

were presented and it is not possible to decide which fractions are truly enriched. Table XI shows that on sonication somewhat less phosphate-dependent glutaminase was released from mitochondria than was glutamate dehydrogenase. This was a consistent finding in these experiments. Similar observations have been made by Wit-Peeters (156) for fatty acid-activating enzymes of guinea pig heart mitochondria. Srere (157) has postulated that the mitochondrial matrix enjoys some element of internal structure and that matrix enzymes are not completely free in solution. Matlib and O'Brien (158) showed compartmentation of Krebs cycle enzymes in mitochondrial matrix. The discrepancy between phosphate-dependent glutaminase and glutamate dehydrogenase release on sonication may well be indicative of some such structure in the matrix. It may also reflect a weak association of phosphate-dependent glutaminase with the inside of the inner-membrane which is disrupted by detergent treatment, but is still partially evident after sonication. Thus, phosphate-dependent glutaminase may be a "peripheral" protein attached very loosely to the inside of inner-membrane of mitochondria.

The presence of phosphate-dependent glutaminase in the mitochondrial matrix or its loose association with the inside of inner-membrane suggests several considerations about its regulation. First, glutamine must traverse at least three membranes to reach phosphate-dependent glutaminase, i.e. the plasma membrane, the outer mitochondrial membrane and the inner mitochondrial membrane. The outer-membrane is generally thought to be freely permeable to small molecules, but the inner-membrane is not. Therefore, the transport of glutamine across the inner-membrane

may require a specific carrier system and presents an additional potential point of metabolic regulation. Second, a matrix localization for phosphate-dependent glutaminase implies that regulation of this enzyme will be effected by the concentration of metabolites in the mitochondrial matrix. Thus, the activity of phosphate-dependent glutaminase will be regulated primarily by substrate concentration and the concentrations of activators (phosphate) and inhibitors (glutamate) in this compartment. Although the concentration of glutamine, phosphate, and glutamate has been measured in rapidly frozen kidney (49) and kidney cortex (38,101), there is no assurance that these measurements represent the *in vivo* concentrations in the mitochondrial matrix. Therefore, information on the relevant concentrations of these metabolites must await the development of techniques for the measurement of *in vivo* levels of metabolites in discrete cell components.

The intracellular distribution of phosphate-independent glutaminase is quite different (Fig. 3). The highest specific activity was invariably found in the microsomal fraction. Similar results have also been reported by Kovacević (55). Since the microsomal fraction is also enriched in NADPH-cytochrome c reductase and 5'-nucleotidase (Fig. 3), the precise location of phosphate-independent glutaminase has not been established by our experiments thus far. Previous workers (51,53,54) have assigned this enzyme to the mitochondria. Thus, in pig kidney, Chappell's group have suggested a location in the mitochondrial outer-membrane (54), or bound to the inside of the inner mitochondrial membrane (53). Similarly, in rat kidney Katunuma *et al.* (51) have suggested that phosphate-independent glutaminase is mainly a mitochondrial enzyme.

While species differences in part may explain the discrepancy between my results and those of Chappell's group, they cannot account for the observations of Katunuma et al. (51). Further, the conditions employed by Chappell's group (53) for phosphate-independent glutaminase assay were quite improper since the activator maleate was omitted. In addition, none of these workers carried out a complete cell fractionation but merely prepared mitochondrial fractions and tested for the presence of phosphate-independent glutaminase. It could be suggested that the enzyme present in their studies represents a microsomal contamination. This possibility is strengthened by my own observations (Table XIV A) with rat kidney and that of Lin and Fishman (139) with mouse kidney, that rather extensive homogenization is required in order to achieve adequate "pinching-off" of endoplasmic reticulum into microsomal vesicles which can be readily separated from mitochondria. In fact, if one homogenizes kidney gently (e.g., so as to prepare intact mitochondria) one risks a high degree of microsomal contamination. In this regard, my own homogenization procedure represents a compromise between the extensive homogenization required to effect a good separation of microsomes from mitochondria and the gentle homogenization necessary to preserve the intactness of mitochondria.

It was especially important to establish the intactness of mitochondria in these studies as it could be possible that rupture of and removal of the outer-membrane and its subsequent appearance in the microsomal fraction could result in an incorrect localization of phosphate-independent glutaminase. The intactness and presence of the mitochondrial outer-membrane in the lysosomal + mitochondrial fraction

TABLE XIV A
 ENZYME ACTIVITIES IN MITOCHONDRIAL PREPARATIONS FROM RAT KIDNEY CORTEX

The enzymes were assayed as described under "Materials and Methods". The mitochondrial fractions were prepared by differential centrifugation ("Materials and Methods") of tissue homogenized to different extents. 100% activity was assigned to the unfractionated homogenate preparations. The values given are from a typical experiment.

Degree of Homogenization (Number of Strokes)	Percentage Enzyme Activity		
	Glutamate Dehydrogenase	Succinate-cytochrome c Reductase	NADPH-cytochrome c Reductase
4	58	56	29
6	63	60	22
8	69	71	10
10	61	66	12

was established by succinate-cytochrome c reductase latency (Table XIII) and by following the distribution of monoamine oxidase (outer-membrane marker, Table XIV). Therefore, the appearance of phosphate-independent glutaminase in the microsomal fraction cannot be due to breakage and removal of the outer mitochondrial membrane.

CHAPTER V

FURTHER STUDIES ON PHOSPHATE-INDEPENDENT GLUTAMINASE

In chapter IV, it was shown that phosphate-dependent glutaminase was located in the matrix compartment of mitochondria whereas phosphate-independent glutaminase was enriched in the microsomal fraction of rat kidney cortex. Since the microsomal fraction represents a heterogeneous collection of membranes derived from endoplasmic reticulum, plasma membrane and the brush border of cells (Fig. 6), the exact cellular localization of phosphate-independent glutaminase is not certain. Therefore, further studies were carried out on the localization and nature of phosphate-independent glutaminase in rat kidney cortex.

INTRACELLULAR DISTRIBUTION OF PHOSPHATE-INDEPENDENT GLUTAMINASE

Table XV shows the distribution of phosphate-independent glutaminase as compared with that of markers of plasma membrane (5'-nucleotidase), brush border (alkaline phosphatase, maltase, γ -glutamyl-transpeptidase) and endoplasmic reticulum (NADPH-cytochrome c reductase) in different subcellular fractions isolated from the rat kidney cortex homogenate. The results are presented in the manner proposed by de Duve et al. (115) in Fig. 12. The intracellular distribution of phosphate-independent glutaminase is similar to that of NADPH-cytochrome c reductase, alkaline phosphatase, maltase, γ -glutamyltranspeptidase and 5'-nucleotidase. The highest specific activity of all these enzymes was found in the microsomal fraction.

BRUSH BORDER LOCALIZATION FOR PHOSPHATE-INDEPENDENT GLUTAMINASE

As shown by differential centrifugation experiments (Fig. 12), the highest specific activity of phosphate-independent glutaminase is present in the microsomal fraction. The different membranes of the microsomal

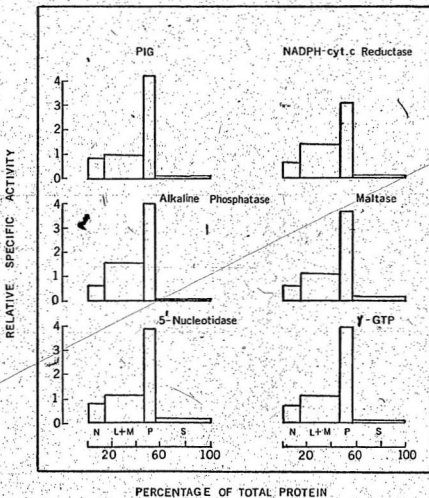
TABLE XV

INTRACELLULAR DISTRIBUTION OF PHOSPHATE-INDEPENDENT GLUCAMINASE AND OTHER ENZYMES OF RAT KIDNEY CORTEX

The enzymes were assayed as described under "Materials and Methods". The absolute values of enzyme activity in the total homogenate (H) are given in micromoles of substrate metabolized per g of kidney cortex (wet weight) per min. The results are given as mean \pm S. D. Each value represents the average of three experiments. H is the total homogenate; N, the nuclear fraction; L + M, the lysosomal + mitochondrial fraction; P, the microsomal fraction and S, the final supernatant.

	Total Homogenate	H	N	L + M	P	S	Total
Protein		100	16.2 \pm 4.6	30.1 \pm 3.9	9.7 \pm 2.6	43.8 \pm 6.2	99.8 \pm 6.4
Phosphate-independent glucaminase	6.1 \pm 1.4	100	13.0 \pm 4.7	27.9 \pm 8.1	40.9 \pm 4.6	2.1 \pm 3.9	83.9 \pm 9.8
Alkaline phosphatase	20.1 \pm 2.1	100	9.3 \pm 3.6	46.1 \pm 9.8	38.4 \pm 6.2	3.4 \pm 2.6	97.2 \pm 6.3
5'-nucleotidase	2.2 \pm 0.3	100	12.8 \pm 7.6	33.4 \pm 1.4	37.6 \pm 3.2	7.6 \pm 5.8	91.4 \pm 8.9
NADPH-cytochrome c reductase	0.9 \pm 0.4	100	10.2 \pm 5.8	41.1 \pm 8.2	29.6 \pm 7.0	5.6 \pm 6.1	86.5 \pm 10.1
Maltase	9.0 \pm 1.4	100	8.5 \pm 4.8	31.6 \pm 4.4	34.5 \pm 4.9	6.0 \pm 4.6	80.7 \pm 8.4
γ -Glutamyltranspeptidase	204.5 \pm 20.1	100	12.2 \pm 7.6	31.8 \pm 4.9	36.7 \pm 6.9	0.4 \pm 0.3	81.1 \pm 9.6

FIG. 12. Distribution pattern of phosphate-independent glutaminase and other enzymes in fractions from rat kidney cortex. The relative specific activities (percentage of total activity/percentage of total protein) of the fractions are plotted on the ordinate. On the abscissa, the fractions are represented by their relative protein content, in the order in which they are isolated, i.e., from left to right. N, nuclear fraction; L + M, lysosomal + mitochondrial fraction; P, microsomal fraction; S, soluble fraction. *FIG.*, phosphate-independent glutaminase; γ -GIP., γ -glutamyltranspeptidase; *NADPH-cyt.-c Reductase*; NADPH-cytochrome c reductase.



fraction could be readily separated from each other by means of gradient centrifugation. Fig. 13 shows the result of a typical experiment in which the microsomal fraction was centrifuged and fractionated as described under "Materials and Methods". The activity profile of phosphate-independent glutaminase exactly paralleled that of alkaline phosphatase, maltase and γ -glutamyltranspeptidase (brush border markers), whereas 5'-nucleotidase and NADPH-cytochrome c reductase were found in the lighter fractions. This indicates that phosphate-independent glutaminase is localized in the brush border of rat kidney cortex. The specific activity of alkaline phosphatase, maltase, γ -glutamyl-transpeptidase and phosphate-independent glutaminase is 15 - 18 times higher in fractions 18 - 20 than in the whole homogenate (Table XVI). This is comparable to the enrichment of alkaline phosphatase and other brush border enzymes observed by other workers in purified brush border preparation (109,160,161).

MEMBRANOUS NATURE OF PHOSPHATE-INDEPENDENT GLUTAMINASE

Table XVII shows the effect of different treatments on the removal of phosphate-independent glutaminase from membranes of the microsomal fraction. Sonication, treatment with physiological concentrations of salt, alterations in pH, or combinations of these treatments did not release appreciable activity of phosphate-independent glutaminase in the supernatant. Most of the activity was retained in the pellet fraction. This indicates that the enzyme is not loosely bound to the brush border membranes. Thus, this enzyme is probably an "integral" membrane protein.

FIG. 13. Distribution pattern of phosphate-independent glutaminase and marker enzymes after centrifugation in a continuous sucrose density gradient. The microsomal fraction was layered over a gradient and centrifuged for 16 h at 25,000 rpm as described under "Materials and Methods". After centrifugation the contents of each tube were collected in 1 ml aliquots which, on the *abscissa*, are represented in the order of their position in the tube, from meniscus (*left*) to bottom (*right*). The *ordinate* represents percentage of the relative enzyme activity recovered in each fraction.

○—○, phosphate-independent glutaminase; □—□, alkaline phosphatase; ●—●, maltase; ●—●, γ -glutamyl-transpeptidase; X—X, NADPH-cytochrome c reductase; ○—○, 5'-nucleotidase; ★—★, protein.

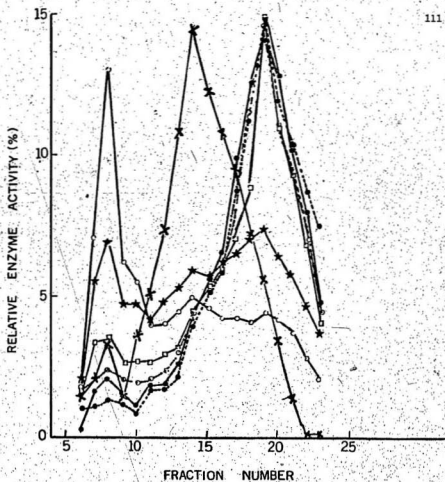


TABLE XVI

SPECIFIC ACTIVITIES OF ORGANELLE SPECIFIC ENZYMES IN STARTING HOMOGENATE,
MICROSOMAL AND BRUSH BORDER FRACTION OF RAT KIDNEY CORTEX

The enzymes were assayed as described under "Materials and Methods". The brush border fraction is composed of fractions 18 to 20 of Fig. 13. All specific activities are given in micromoles per min per mg protein.

Enzyme	Specific Enzyme Activity			Relative Specific Activity (C/A)
	Homogenate (A)	Microsomal Fraction (B)	Brush Border Fraction (C)	
Alkaline Phosphatase	0.117	0.461	1.760	15
Phosphate-Independent Glutaminase	0.024	0.078	0.440	18
Maltase	0.052	0.209	0.806	15
γ -Glutamyltranspeptidase	1.191	5.148	20.130	17

TABLE XVII

EFFECT OF DIFFERENT TREATMENTS ON THE REMOVAL OF PHOSPHATE-INDEPENDENT
GLUTAMINASE FROM MEMBRANES OF THE MICROSOMAL FRACTION

The treatments were given as described under "Materials and Methods". All specific activities are given in millimicroles per min per mg protein. Total activities are based on unfractionated preparation.

Treatment	Unfractionated Preparation	Pellet	Supernatant	Recovery (%)
Sonication				
Specific activity	82.2	98.6	32.5	
Total activity (%)	100	85	13	98
KCl				
Specific activity	80.1	92.3	15.5	
Total activity (%)	100	95	5	100
KCl plus sonication				
Specific activity	85.0	86.1	19.1	
Total activity (%)	100	75	7	82
pH 5.5				
Specific activity	54.2	55.3	0	
Total activity (%)	100	94	0	94
pH 5.5 plus sonication				
Specific activity	60.3	67.2	28.7	
Total activity (%)	100	95	8	103
pH 8.5				
Specific activity	59.6	66.5	16.7	
Total activity (%)	100	92	5	97
pH 8.5 plus sonication				
Specific activity	56.4	66.2	11.8	
Total activity (%)	100	94	5	99

pH DEPENDENCE OF THE PHOSPHATE-INDEPENDENT GLUTAMINASE ACTIVITY

The effect of pH on the enzyme activity was studied in the presence and absence of maleate. It was found that optimum pH for enzyme activity was at pH 7.4 (Fig. 14) in the presence of maleate whereas in the absence of maleate the activity was low and almost independent of pH in the range of 5.5 to 9.0.

EFFECT OF SUBSTRATE CONCENTRATION ON PHOSPHATE-INDEPENDENT GLUTAMINASE ACTIVITY

Fig. 15(A) shows the effect of different concentrations of glutamine on phosphate-independent glutaminase in the absence and presence of maleate. There is a slight inhibition of enzyme activity at higher substrate concentration. The K_m for glutamine was also determined by the method of Lineweaver and Burk (Fig. 15(B)). The values obtained were 1.6 ± 0.2 mM ($n = 3$) in the presence of maleate, and 1.2 ± 0.2 mM ($n = 3$) in its absence.

PREINCUBATION OF MICROSOMES WITH MALEATE: EFFECT ON PHOSPHATE-INDEPENDENT GLUTAMINASE ACTIVITY

Since maleate stimulates phosphate-independent glutaminase, it was of interest to see if preincubation of the microsomal fraction with maleate and subsequent removal of the maleate results in a permanent effect on the enzyme activity. Table XVIII shows the result of such an experiment. It is clear that preincubation of microsomal fraction with maleate does not have any effect on phosphate-independent glutaminase activity and it is the presence of maleate in the reaction medium which affects enzyme activity.

FIG. 14. pH activity profile of phosphate-independent glutaminase. pH was maintained by using 20 mM MES, 20 mM Tris and 20 mM HEPES. O—O, activity in the presence of 60 mM maleate; ●—●, activity in the absence of maleate.

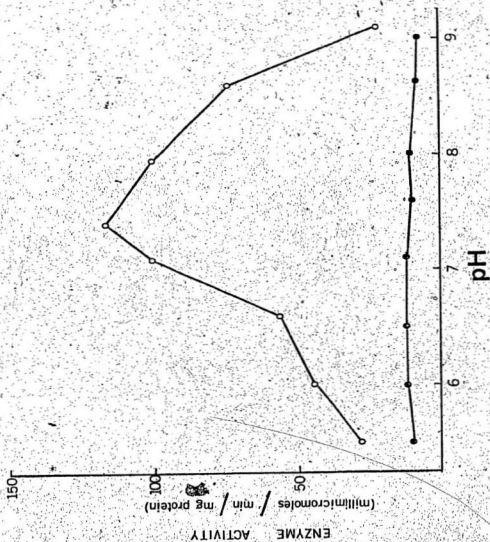


FIG. 15(A). Activity of phosphate-independent glutaminase at various glutamine concentrations. O—O, activity in the presence of 60 mM maleate; ●—●, activity in absence of maleate. The results presented are from a typical experiment. The enzyme activity was assayed at pH 7.4.

FIG. 15(B). Double reciprocal plots of phosphate-independent glutaminase as a function of glutamine concentration in the presence and absence of maleate. The results presented are from a typical experiment.

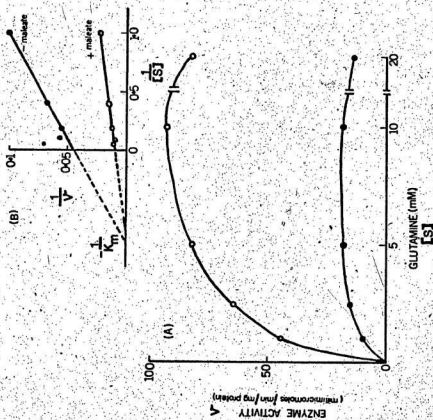


TABLE XVIII
EFFECT OF PREINCUBATION WITH MALEATE ON THE
PHOSPHATE-INDEPENDENT GLUTAMINASE ACTIVITY OF RAT KIDNEY CORTEX

The microsomal fraction was incubated at 37° for 30 min in the absence or presence of 60 mM maleate (pH 7.4). After cooling, the suspension was centrifuged at 105,000 x g for 60 min and the resulting pellet was washed once before suspending in homogenizing medium. The enzyme activity was assayed at pH 7.5 as described under "Materials and Methods". All specific activities are given in millimicromoles per min per mg protein.

Treatment	Specific Enzyme Activity	
	- Maleate	+ Maleate
None	19.0	97.2
Preincubation		
- maleate	19.5	97.8
+ maleate	17.1	100.0

EFFECT OF DIFFERENT COMPOUNDS ON PHOSPHATE-INDEPENDENT GLUTAMINASE

Phosphate-independent glutaminase activity is strongly activated by maleate. However, I have found no report of the existence of maleate in animals (though it is often a constituent of wine). Therefore, naturally-occurring carboxylic acids were examined for their ability to activate this enzyme. Table XIX shows the effect of different carboxylic acids on phosphate-independent glutaminase. It is clear that at 20 mM concentration of acetate, there is 2.6-fold increase in the enzyme activity, whereas no substantial activation was observed in the presence of other carboxylic acids. Similarly, no activation was observed in the presence of acetoacetate or β -hydroxybutyric acid (data not shown). Thus, the excretion of large quantities of these acids as, for example, in diabetic ketoacidosis (1) does not stimulate ammonia production by this enzyme.

DISCUSSION

The studies reported in this chapter clearly indicate that phosphate-independent glutaminase is located in the brush borders of rat kidney cortex. Curthoys and Lowry (68) have shown that this enzyme is largely present in the cells of the proximal straight tubules. Although brush borders are more prevalent in the convoluted portion of this tubule, appreciable numbers are also seen in the straight portion (162). It is, therefore, possible that phosphate-independent glutaminase is located preferentially in the brush borders of the proximal straight tubule. It was important to demonstrate that this enzyme was not loosely bound to the brush border membranes. It is well known that artifactual localizations can result from such associations; for example, glutamine

TABLE XIX
EFFECT OF DIFFERENT CARBOXYLIC ACIDS

ON PHOSPHATE-INDEPENDENT GLUTAMINASE ACTIVITY

The enzyme was assayed as described under "Materials and Methods".
All the carboxylic acids were used at indicated concentrations.

Compound Added	Percentage Enzyme Activity	
	Final Concentration of Compound	
	(20 mM)	(2 mM)
None	100	100
Maleic acid	440	170
Malic acid	140	100
Sodium acetate	260	105
Succinic acid	140	105
Aspartic acid	80	100
Lactic acid	140	100
Fumaric acid	130	75
Pyruvic acid	100	120
Citric acid	30	105
α -ketoglutaric acid	170	75

synthetase has been shown to be specifically bound to rat liver microsomes and could be quantitatively removed by physiological concentrations of salt (146). Sonication would remove loosely bound material and, since it disrupts membrane vesicles, would also release trapped enzymes. Physiological concentrations of salt and alterations in pH would be expected to remove enzymes that were electrostatically attached. That none of these treatments alone or in combination resulted in the removal of phosphate-independent glutaminase clearly indicates that this is a truly membranous enzyme.

It has been demonstrated both histochemically and biochemically that alkaline phosphatase (160,161,163) and γ -glutamyltranspeptidase (107-109) activities in the kidney are concentrated in the brush border membrane of the proximal tubule cells. Wilfong and Neville (161) showed a 16-fold increase in specific activity of alkaline phosphatase in preparations of rat kidney brush border membrane relative to the starting homogenate. Electron microscopic examination of the preparation of Wilfong and Neville showed it to be extremely enriched in microvilli. A similar preparation showed an 18-fold increase in γ -glutamyltranspeptidase activity (109).

γ -Glutamyltranspeptidase, maltase and phosphate-independent glutaminase appeared predominantly, if not exclusively, in the same fractions as alkaline phosphatase (Fig. 13). In each instance, the enzyme was enriched (compared with the cortical homogenate) 15 - 18 fold. Other workers have reported comparable enrichment value for these enzymes (Table XX). Glossmann and Neville (108) observed that

TABLE XX
ENRICHMENT VALUES FOR KIDNEY BRUSH BORDER ENZYMES

Enrichment values are expressed as the ratio of the specific activity in the brush border preparation to that of the kidney homogenate. Authors: JK, Kime and Kime-Saffran (160) ; WN, Wilfong and Neville (161) ; GN, Glosmann and Neville (108) ; QR, Quirk and Robinson (164) ; GK, George and Kenny (109) ; JK, this thesis.

	Authors	KK	WN	GN	QR	CK	JK
	Animals	Rat	Rat	Rat	Rabbit	Rabbit	Rat
Alkaline phosphatase		12	16	16	19	15	15
Maltase		-	-	17	16	22	15
γ -Glutamyltranspeptidase		-	-	5	-	18	17
Phosphate-independent glutaminase		-	-	-	-	-	18

γ -glutamyltranspeptidase activity was only 5-fold enriched in contrast with the high enrichment in my studies and that of George and Kenny (109). The enrichment of phosphate-independent glutaminase is very much comparable with other brush border enzymes.

CHAPTER VI

 γ -GLUTAMYL TRANSFERRING ACTIVITIES OF RAT KIDNEY CORTEX

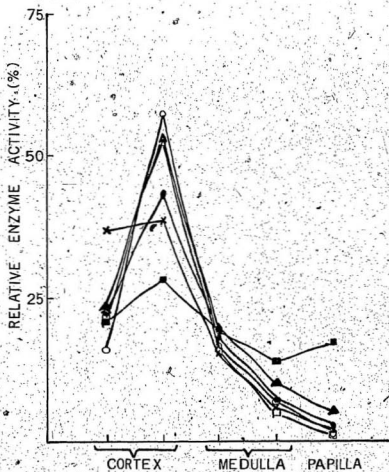
In the last chapter, it was shown that phosphate-independent glutaminase is truly a membranous enzyme and is located in the brush border membranes of rat kidney cortex. The reaction catalyzed by phosphate-independent glutaminase resembles the hydrolytic reaction of γ -glutamyltranspeptidase which is shown to be mainly concentrated in the brush border membranes of the proximal tubule (106-109). This raises the possibility that both the reactions are catalyzed by the same enzyme. There are a variety of other enzymes which are shown to catalyze the transfer of γ -glutamyl moiety to various acceptors in rat kidney. Therefore, experiments were carried out to establish whether these activities are catalyzed by separate enzymes or whether some of them represent different catalytic manifestations of the same protein.

REGIONAL DISTRIBUTION OF ENZYMES

The regional distribution of the various γ -glutamyltransferring activities as well as of the brush border marker enzyme, alkaline phosphatase, was determined (Fig. 16). It is clear that alkaline phosphatase activity is high in both the outer and inner cortex region. This finding is consistent with histological studies which indicate that this enzyme is distributed in the brush border membranes of both proximal convoluted and proximal straight tubule cells (163). The highest activities of γ -glutamyltranspeptidase and phosphate-independent glutaminase was observed in the inner cortex region. The localization of γ -glutamyltranspeptidase appears to be inconsistent with its reported histochemical localization in the proximal convoluted tubular cells (107). On the contrary, Curthoys and Lowry (58), with quantitative microanalysis have shown that phosphate-independent glutaminase is primarily present in

proximal straight tubular cells. It seems possible that the histochemical technique did not reflect a proper localization of γ -glutamyltranspeptidase and that this enzyme is located in both proximal convoluted and straight tubule cells but primarily in the straight portion. All the other enzyme examined had their highest activities in the inner cortex. In addition, a peak of phosphate-independent glutaminase activity was apparent in the papillae. This papillary activity must be quite distinct from the other enzymes. However, it was not considered further and the subsequent experiments were all carried out with cortical preparations.

FIG. 16. Regional distribution of a brush border membrane marker (alkaline phosphatase) and of other enzyme activities in rat kidney. A cone of kidney tissue was cut in such a way that its base consisted solely of cortical tissue and its apex consisted solely of papillary region. The cone was cut in outer cortex, inner cortex, outer medulla, inner medulla and papilla starting from base to top (left to right). The slices from the same region were pooled from 3 to 4 rats and homogenized. The enzyme activities were measured as described under "Materials and Methods". □—□, γ -glutamyltranspeptidase; ×—×, alkaline phosphatase; ■—■, phosphate-independent glutaminase; ○—○, γ -glutamyltransferase; ▲—▲, glutamine synthetase; ●—●, glutamylhydroxamase.



LACK OF IDENTITY OF GLUTAMINE SYNTHETASE WITH PHOSPHATE-INDEPENDENT
GLUTAMINASE

It has been suggested that phosphate-independent glutaminase is a partial activity of glutamine synthetase (91,113). I, therefore, carried out experiments to see whether these activities could be separated. It is known that rat liver glutamine synthetase can be readily removed from microsomes by treatment with either EDTA or with sodium chloride (146). Therefore, rat kidney cortex microsomes were subjected to these treatments so as to see whether glutamine synthetase and phosphate-independent glutaminase activities could be dissociated. Table XII shows the effects of different treatments on the removal of glutamine synthetase and phosphate-independent glutaminase from membranes of the microsomal fraction. Treatment of microsomes with a physiological concentration of sodium chloride or with 0.054 M EDTA results in a release of glutamine synthetase activity into the supernatant whereas most of the phosphate-independent glutaminase activity was found in the pellet fraction. These results indicate that phosphate-independent glutaminase activity of rat kidney cortex cannot be attributed to glutamine synthetase.

LACK OF IDENTITY OF γ -GLUTAMYLTRANSFERASE WITH GLUTAMINE SYNTHETASE

Since purified glutamine synthetase also catalyzes the γ -glutamyl-transferase reaction (114), this activity of tissues has been attributed to glutamine synthetase. Therefore, the subcellular localization of these enzymes was studied in kidney cortex. Table XXII shows very different distributions of these two enzymes in different subcellular fractions. Glutamine synthetase was enriched in the microsomal fraction. However,

TABLE XXI
EFFECT OF DIFFERENT TREATMENTS ON THE REMOVAL OF GLUTAMINE SYNTHETASE AND
PHOSPHATE-INDEPENDENT GLUTAMINASE FROM MEMBRANES OF THE MICROSOPL FRACTION

The treatments were given as described under "Materials and Methods". All specific activities are given in millimicroles per min per mg protein. Total activities are based on unfractionated preparation. The values given are from a typical experiment.

Treatment	Glutamine Synthetase				Phosphate-Independent Glutaminase			
	Unfractionated Preparation	Pellet	Supernatant	Recovery (%)	Unfractionated Preparation	Pellet	Supernatant	Recovery (%)
None								
Specific activity	111	118	64		145	166	0	
Total activity (%)	100	89	11	100	100	95	0	96
NaCl								
Specific activity	126	37	337		159	169	0	
Total activity (%)	100	28	84	110	100	93	0	93
EDTA								
Specific activity	185	47	468		179	182	0	
Total activity (%)	100	24	88	112	100	91	0	91

TABLE XXII
 PERCENTAGE RECOVERIES AND SPECIFIC ACTIVITIES OF GLUTAMINE SYNTHETASE AND
 γ -GLUTAMYLTRANSFERASE AFTER SUBCELLULAR FRACTIONATION OF RAT KIDNEY CORTEX

The different fractions were prepared and assayed for enzyme activities as described under "Materials and Methods". All specific activities are given in millimicroles per min per mg protein.

Fractions	Glutamine Synthetase		γ -Glutamyltransferase	
	Total Activity (%)	Specific Activity	Total Activity (%)	Specific Activity
Homogenate	100	37	100	651
Nuclear	13.8	29	6.4	252
Lysosomal + mitochondrial	40.3	59	11.4	277
Microsomal	34.7	142	7.5	865
Soluble	4.1	3	71.3	1,327
Recovery	92.9		96.6	

γ -glutamyltransferase was enriched in both microsomal and supernatant fractions. It is clear that the soluble γ -glutamyltransferase activity is distinct from glutamine synthetase, but it is possible that all of the particulate activity could be due to glutamine synthetase. That this is not the case was demonstrated by experiments in which microsomes were treated with NaCl as described in Table XXI. This treatment removed almost all of the glutamine synthetase, but only 14% of the γ -glutamyltransferase. Thus, the small fraction of γ -glutamyltransferase activity removed by salt treatment may be due to glutamine synthetase, but in addition, it is clear that there is both a soluble γ -glutamyltransferase and a particulate γ -glutamyltransferase.

LACK OF IDENTITY OF PHOSPHATE-INDEPENDENT GLUTAMINASE WITH γ -GLUTAMYLTRANSFERASE

The results reported in Table XXI indicate that glutamine synthetase and phosphate-independent glutaminase activities are due to two different proteins. However, it remains possible that γ -glutamyltransferase and phosphate-independent glutaminase activities are catalyzed by the same protein. The data in Table XXII demonstrating that γ -glutamyltransferase is largely a soluble enzyme precludes this conclusion since phosphate-independent glutaminase is a particulate enzyme.

IDENTITY OF PHOSPHATE-INDEPENDENT GLUTAMINASE AND GLUTAMINOHYDROXAMASE

Lamar (47) has described an enzymatic activity present in rat kidney microsomes which hydrolyses γ -glutamylhydroxamate and glutamine. Therefore, experiments were carried out to see whether this activity is

similar to that of phosphate-independent glutaminase. Table XXIII shows the results of experiments where the effects of maleate on the hydrolysis of various γ -glutamyl compounds was studied. Glutamine, glutathione, γ -glutamyl-p-nitroanilide and γ -glutamylhydroxamate were all readily hydrolyzed by kidney cortex microsomes and the hydrolysis was, in each case, stimulated by maleate. The appearance of glutamic acid demonstrates that hydrolysis takes place and γ -glutamyl residues are transferred to water.

Since the hydrolysis of γ -glutamylhydroxamate is stimulated by maleate (Table XXIII), the possibility presents itself that glutamohydroxamase may be identical to phosphate-independent glutaminase. Therefore, competition experiments were carried out between glutamine and γ -glutamylhydroxamate, both substrates being at saturating concentrations. If the hydrolytic reactions are carried out by two different proteins, then the total activity in the presence of glutamine plus γ -glutamylhydroxamate should be equal to the sum of enzymatic activity with glutamine and γ -glutamylhydroxamate alone. However, if the hydrolytic reactions are carried out by the same protein then the total activity in the presence of glutamine and γ -glutamylhydroxamate should be less than the sum of the activity with glutamine and γ -glutamylhydroxamate alone. Therefore, the effect of substrate concentration on glutamohydroxamase activity was studied so as to establish saturating concentration for the hydrolysis of this substrate.

Fig. 17 (A) shows the effect of different concentrations of γ -glutamylhydroxamate on glutamohydroxamase activity in the absence and

TABLE XXIII

EFFECT OF MALEATE ON THE FORMATION OF GLUTAMATE FROM
VARIOUS γ -GLUTAMYL COMPOUNDS BY RAT KIDNEY CORTEX MICROSOMES

The reaction mixture (final volume 1.0 ml) contained: HEPES (20 mM; pH 7.6), EDTA (0.2 mM), γ -glutamyl compound (glutamine 10 mM; glutathione 10 mM; γ -glutamyl-p-nitroanilide 5 mM; or γ -glutamyl-hydroxamate 5 mM) without or with maleate (60 mM). The reaction was initiated by the addition of an aliquot of microsomal fraction. The reaction was stopped after 10 min with 0.05 ml of 10 N HCl. The proteins were centrifuged and an aliquot of supernatant was used for the measurement of glutamate.

γ -Glutamyl Compound	Enzyme Activity ^b		
	Without Maleate (A)	With Maleate (B)	Ratio B/A
Glutamine	18	85 (100) ^a	4.72
Glutathione	76	128 (151)	1.68
γ -Glutamyl-p-nitroanilide	47	129 (152)	2.74
γ -Glutamylhydroxamate	16	66 (77)	4.12

^aThe values in parentheses are activities relative to glutamine.

^bMillimicromoles of glutamate formed per min per mg protein.

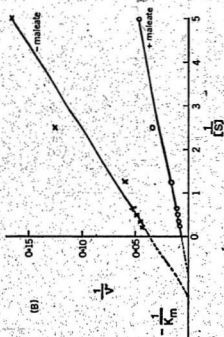
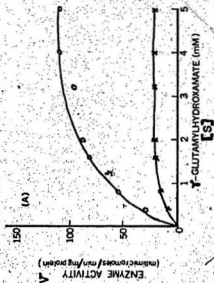


FIG. 17(A). Activity of glutamylhydroxamase at various γ -glutamylhydroxamate concentrations. O—O, activity in the presence of 60 mM maleate; X—X, activity in the absence of maleate. The results presented are from a typical experiment.

FIG. 17(B). Double reciprocal plots of glutamylhydroxamase as a function of γ -glutamylhydroxamate concentration in the presence and absence of maleate. The results are from a typical experiment.

and presence of maleate. The K_m for γ -glutamylhydroxamate was also determined by the method of Lineweaver and Burk (Fig. 17 (B)). The values obtained were 0.80 ± 0.13 mM ($n = 3$) in the absence of maleate, and 0.98 ± 0.20 mM ($n = 3$) in its presence. Table XXIV shows the hydrolysis of glutamine and γ -glutamylhydroxamate by the rat kidney cortex microsomal fraction. The total enzyme activity in the presence of glutamine plus γ -glutamylhydroxamate is less than that of the sum of the activities in the presence of glutamine and γ -glutamylhydroxamate alone. These results are consistent with the proposition that two substrates are being hydrolyzed by the same protein. In other experiments, glutamine, glutathione, γ -glutamyl-p-nitroanilide and different combinations of these γ -glutamyl donors were used, all at saturating concentrations, as substrates. Table XXV shows the result of these experiments. The total enzyme activity in the presence of any two γ -glutamyl donors is always less than that of the sum of their activities, when assayed individually. These results suggest that there exists in kidney cortex microsomes a single enzymatic activity that can hydrolyse different γ -glutamyl compounds in a maleate-stimulated fashion. This activity is identical to phosphate-independent glutaminase which is shown to be localized on the brush border membrane.

IDENTITY OF PHOSPHATE-INDEPENDENT GLUTAMINASE AND γ -GLUTAMYLTRANSPEPTIDASE:
EFFECT OF HEAT TREATMENT ON PHOSPHATE-INDEPENDENT GLUTAMINASE AND
 γ -GLUTAMYLTRANSPEPTIDASE ACTIVITIES

Katunuma et al. (51) demonstrated that phosphate-independent glutaminase is relatively resistant to heat treatment. Therefore, I compared the effects of heat treatment of the microsomal fraction on

TABLE XXIV
HYDROLYSIS OF GLUTAMINE AND γ -GLUTAMYLHYDROXAMATE
BY RAT KIDNEY CORTEX MICROSOMES

The reaction mixture (final volume 1.0 ml) contained: HEPES (20 mM; pH 7.6), EDTA (0.2 mM), maleate (60 mM) and γ -glutamyl compound at saturating concentrations (glutamine 10 mM; γ -glutamylhydroxamate 5 mM). The reaction was initiated by the addition of an aliquot of microsomal fraction. The reaction was stopped after 10 min with 0.05 ml of 10 N HCl. The proteins were centrifuged and an aliquot of supernatant was used for the measurement of glutamate.

γ -Glutamyl Compound	Enzyme Activity ^b
Glutamine	94 (100) ^a
γ -Glutamylhydroxamate	53 (55)
Glutamine plus γ -Glutamylhydroxamate	83 (88)

^aThe values in parentheses are activities relative to glutamine.

^bMillimicromoles of glutamate formed per min per mg protein.

TABLE XXV
HYDROLYSIS OF VARIOUS γ -GLUTAMYL COMPOUNDS
BY RAT KIDNEY CORTEX MICROSOMES

The reaction mixture (final volume 1.0 ml) contained: HEPES (20 mM; pH 7.6), EDTA (0.2 mM), maleate (60 mM) and γ -glutamyl compound at saturating concentrations (glutamine 10 mM; glutathione 10 mM; γ -glutamyl-p-nitroanilide 5 mM). The reaction was initiated by the addition of an aliquot of microsomal fraction. The reaction was stopped after 10 min with 0.05 ml of 10 N HCl. The proteins were centrifuged and an aliquot of supernatant was used for the measurement of glutamate.

γ -Glutamyl Compound	Enzyme Activity	
	Specific Activity ^b	Relative Activity
Glutamine	83	(100) ^a
Glutathione	167	(201)
γ -Glutamyl-p-nitroanilide	145	(174)
Glutamine plus glutathione	141	(169)
Glutamine plus γ -glutamyl-p-nitroanilide	167	(201)
Glutathione plus γ -glutamyl-p-nitroanilide	132	(158)

^aThe values in parentheses are relative to glutamine.

^bMillimicromoles of glutamate formed per min per mg protein.

both phosphate-independent glutaminase and γ -glutamyltranspeptidase activities. Tables XXVI to XXVIII show the results of these experiments. It is clear that both enzyme activities are resistant to short heat treatment at temperatures up to 55° (Table XXVI) whereas at 60° and 65°, there is a comparable loss of both enzyme activities. At 70° there is complete loss of both the enzyme activities (Table XXVIII). There is comparable loss of both enzyme activities at 60° for at least 60 min (Table XXVII).

RELEASE OF ENZYMES AFTER TREATMENT OF THE MICROSOMAL FRACTION WITH PAPAIN

Table XXIX shows the effect of papain on the release of different enzymes from membranes of the microsomal fraction. Treatment of microsomes with papain for different time periods results in a comparable release of phosphate-independent glutaminase and γ -glutamyltranspeptidase activities into the supernatant, whereas maltase, another brush border enzyme, was released into the supernatant at quite a different rate. The recovery of these enzymes after papain treatment was 94-97% of that present before treatment. The results with maltase suggest that the parallel release of phosphate-independent glutaminase and γ -glutamyltranspeptidase is not due to a generalized destruction of brush border membranes.

CHROMATOGRAPHY OF PAPAIN SOLUBILIZED MICROSOMAL FRACTION

The results reported in Tables XXVI - XXIX failed to dissociate phosphate-independent glutaminase from γ -glutamyltranspeptidase. Therefore, purification of these activities was attempted. The solubilized proteins obtained after papain treatment of the microsomal fraction were

TABLE XXVI

EFFECTS OF HEAT TREATMENT ON PHOSPHATE-INDEPENDENT
GLUTAMINASE AND γ -GLUTAMYLTRANSEPTIDASE ENZYME ACTIVITIES

The microsomal fraction was heated at 55° for the indicated time periods, immediately cooled to 0° and then aliquots were used for measurement of enzyme activities as described under "Materials and Methods".

Time (Min)	Percentage Enzyme Activity	
	Phosphate-Independent Glutaminase	γ -glutamyltranspeptidase
0	100	100
10	97	100
20	97	99
40	96	94
60	93	90

TABLE XXVII
EFFECTS OF HEAT TREATMENT ON PHOSPHATE-INDEPENDENT
GLUTAMINASE AND γ -GLUTAMYLTRANSPEPTIDASE ENZYME ACTIVITIES

The microsomal fraction was heated at 60° for the indicated time periods, immediately cooled to 0° and then aliquots were used for measurement of enzyme activities as described under "Materials and Methods".

Time (Min)	Percentage Enzyme Activity	
	Phosphate-Independent Glutaminase	γ -glutamyltranspeptidase
0	100	100
2	96	94
5	90	90
10	88	86
20	82	80
30	79	77
40	78	72
60	76	73

TABLE XVIII

EFFECTS OF HEAT TREATMENT ON PHOSPHATE-INDEPENDENT
GLUTAMINASE AND γ -GLUTAMYLTRANSEPTIDASE ENZYME ACTIVITIES

The microsomal fraction was heated for 10 min at the indicated temperatures, immediately cooled to 0° and then aliquots were used for measurement of enzyme activities as described under "Materials and Methods".

Treatment (Temperature)	Percentage Enzyme Activity	
	Phosphate-Independent Glutaminase	γ -glutamyltranspeptidase
None	100	100
50°	100	100
55°	97	96
60°	88	83
65°	24	19
70°	0	0

TABLE XXIX

EFFECT OF PAPAIN ON THE RELEASE OF DIFFERENT ENZYMES FROM MEMBRANES OF THE MICROSOMAL FRACTION

The microsomal fraction was treated with papain as described under "Material and Methods". At indicated time period, samples were withdrawn, immediately cooled to 0° and then centrifuged at 105,000 x g for 60 min. The supernatant was removed and the pellet was resuspended before enzyme assays.

Incubation Time (Min)	Percentage Enzyme Activity					
	Phosphate-Independent Glutaminase		γ-Glutamyltranspeptidase		Maltase	
	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant
0	92	8	95	5	51	49
10	88	12	91	9	22	78
20	84	16	86	14	17	83
30	75	25	79	21	17	83
40	72	28	73	27	14	86
60	63	37	61	39	11	89

chromatographed on a column of sephadex G-100. The results obtained from such an experiment are illustrated in Fig. 18. It is clear that both phosphate-independent glutaminase and γ -glutamyltranspeptidase activities appeared in the same fractions. The peak of blue dextran (void volume) appeared in fraction 8.

Further purification experiments were not carried out because at this stage, reports from two other laboratories (58,59) demonstrated that highly purified preparations of γ -glutamyltranspeptidase also carried out the phosphate-independent glutaminase reaction. However, I carried out one further set of experiments to investigate the effect of glutamine on γ -glutamyltranspeptidase kinetics. If phosphate-independent glutaminase and γ -glutamyltranspeptidase activities are catalyzed by the same active site, then competition between the two γ -glutamyl compounds should be demonstrable. Table XXX shows the result of one such experiment. Glutamine inhibits the γ -glutamyltranspeptidase activity. The effect of two concentrations of glutamine on γ -glutamyltranspeptidase activity at different substrate concentrations was then studied in order to understand the nature of the inhibition. Fig. 19 shows the result of such an experiment. The effect of glutamine is to increase the K_m of γ -glutamyltranspeptidase for γ -glutamyl-p-nitroanilide without affecting the V_{max} . Thus, the effect of glutamine is to compete with γ -glutamyl-p-nitroanilide. Hence, I concluded that phosphate-independent glutaminase and γ -glutamyltranspeptidase represent different catalytic activities of the same active site.

FIG. 18. Chromatography of papain solubilized microsomal fraction on Sephadex G-100 column. The microsomal fraction was incubated at 37° with papain for 60 min and a sample (2 ml) of the 105,000 x g supernatant was applied to the column and eluted with buffer as described under "Materials and Methods". ●-●, γ -glutamyltranspeptidase; X-X, phosphate-independent glutaminase.

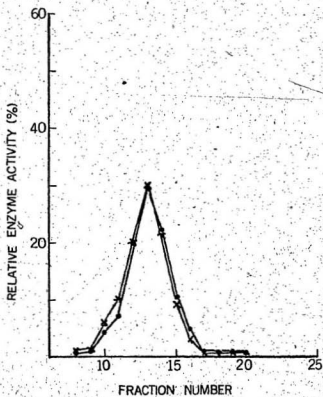


TABLE XXX

EFFECT OF GLUTAMINE ON γ -GLUTAMYLTRANSPEPTIDASE ACTIVITY

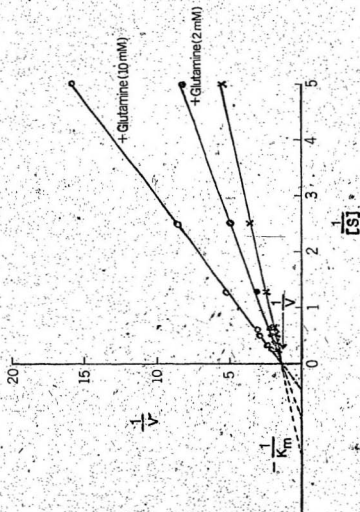
The enzyme activity was measured as described under "Materials and Methods". The concentration of glutamine was 10 mM.

Compounds Added	Enzyme Activity	
	Specific Activity ^b	Relative Activity
None	31.64	(100) ^a
Glutamine	20.76	(65.6)

^aThe values in parentheses are relative to one with no glutamine.

^bMicromoles of p-nitroaniline formed per min per mg protein.

FIG. 19. Effect of Glutamine on γ -Glutamyltranspeptidase
Activity.



CONCLUSIONS

The data presented in this chapter permits the following conclusions to be drawn regarding the identity of the various γ -glutamyl transferring activities of rat kidney cortex.

1. Phosphate-independent glutaminase, γ -glutamyltranspeptidase and glutamylhydroxamase activities are all due to a single enzyme protein. This enzyme also catalyzes the hydrolysis of various γ -glutamyl compounds in a maleate-stimulated fashion. The physiological reaction is probably that of γ -glutamyltranspeptidase and its function may be in amino acid transport as has been suggested by Meister (165). However, a role for this enzyme in ammonia production is not excluded.

2. Glutamine synthetase is quite separate from γ -glutamyltranspeptidase protein. Its subcellular localization *in vivo* is probably in the cytoplasm and its microsomal localization after fractionation in media of low ionic strength is probably an artifact. Its physiological function is the synthesis of glutamine, but the cell type (s) in which it occurs is not known.

3. There are two major γ -glutamyltransferase activities. The major activity is soluble and is quite distinct from glutamine synthetase. A microsomal activity which may also be dissociated from the glutamine synthetase is also demonstrated. The reaction catalyzed *in vivo* by these enzymes and their physiological function remains unclear.

CHAPTER VII
GENERAL DISCUSSION

Ammonia excretion is important in the removal of excess acid from the body and hence, for the maintenance of acid-base balance. The excretion of strong acids by renal tubules is limited by the inability of the kidney to maintain concentration gradients of protons between urine and blood greater than 800/1 (1). The secretion of the free base, ammonia (NH_3), into tubular fluid removes tubular fluid protons (by formation of ammonium (NH_4^+)) and thus, facilitates continued acidification without reaching the limiting proton gradient. For this mechanism to be effective, ammonia secretion should occur at the same site as acidification. In fact, micropuncture studies in normal and acidotic rats have shown that the proximal tubule is responsible for about 70% of excreted ammonia and the distal tubule for most of the balance (22,23). In fact, as much as 50% of urinary ammonia excretion by both normal and acidotic rat kidney occurs in the proximal convoluted tubule (22). The work of Curthoys and Lowry (68) has shown that phosphate-dependent glutaminase activity is normally high in the distal tubules, but during acidosis increases to very high levels in the proximal convoluted tubules. Phosphate-independent glutaminase, by comparison, has high activity only in the proximal straight tubule. It seems likely, therefore, that phosphate-dependent glutaminase in the proximal convoluted tubules may be responsible for the production of as much as half of the total ammonia excreted while the balance may be provided by activities such as phosphate-dependent glutaminase in the distal tubules and phosphate-independent glutaminase in the proximal straight tubules.

A study of the regulation of ammonia production clearly necessitates

a precise intracellular localization of these enzymes. The results presented in this thesis clearly showed that phosphate-dependent glutaminase is located within the matrix compartment of mitochondria of liver and kidney cortex of rat. The enzyme could be loosely attached to the inside of inner-membrane of mitochondria. This suggests several considerations about phosphate-dependent glutaminase regulation. First, glutamine must traverse at least three membranes to reach phosphate-dependent glutaminase, i.e., the plasma membrane, the outer mitochondrial membrane, and the inner mitochondrial membrane. The outer-membrane is generally thought to be freely permeable to small molecules, but the inner-membrane is not. Therefore, the transport of glutamine across the inner-membrane may require a specific carrier system and presents an additional potential point of metabolic regulation. Second, a matrix localization for phosphate-dependent glutaminase implies that regulation of this enzyme will be effected by the concentration of metabolites in the mitochondrial matrix. Thus, the activity of phosphate-dependent glutaminase will be regulated primarily by substrate concentration and the concentrations of activators (phosphate) and inhibitors (glutamate). Although the concentration of glutamine, phosphate, and glutamate has been measured in rapidly frozen kidney (49) and kidney cortex (38,101), there is no assurance that these measurements represent the *in vivo* concentrations in the mitochondrial matrix. Therefore, information on the relevant concentrations of these metabolites must await the development of techniques for the measurement of *in vivo* levels of metabolites in discrete cell components.

On the other hand, the intracellular distribution of phosphate-

independent glutaminase is quite different. This enzyme is shown to be located in the brush border membrane and appeared to be identical to that of γ -glutamyltranspeptidase. It is possible that γ -glutamyltranspeptidase - phosphate-independent glutaminase may play a physiological role in ammoniagenesis, besides the suggested role for γ -glutamyltranspeptidase in amino acid transport (165). The localization of γ -glutamyltranspeptidase - phosphate-independent glutaminase in the brush border membranes may be particularly advantageous for ammonia production since (i) 70% of the ammonia excretion occurs in the proximal tubule in the rat (22), (ii) it requires the minimum distance for ammonia diffusion between the site of its formation and its discharge into the tubular fluid, and (iii) it affords a locus where there would be adequate supplies of glutamine since reabsorption of amino acids occurs along the entire length of the proximal tubule.

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PUBLICATIONS ARISING FROM THIS WORK

Papers: -

1. Jawahar Kalra and John T. Brosnan. The Subcellular Localization of Glutaminase Isoenzymes in Rat Kidney Cortex. *J. Biol. Chem.* 249, 3255 (1974).
2. J. Kalra and John T. Brosnan. Localization of Phosphate-Independent Glutaminase in the Brush Border of Rat Kidney Cortex. *Can. J. Biochem.* 52, 762 (1974).
3. J. Kalra and J. T. Brosnan. Localization of Glutaminase in Rat Liver. *FEBS Letters* 37, 325 (1973).
4. J. Kalra and John T. Brosnan. γ -Glutamyl Transferring Activities of Rat Kidney Cortex (submitted to *Biochim. Biophys. Acta*).

Abstracts: -

1. J. Kalra and J. T. Brosnan. Similarities Between γ -Glutamyl-transpeptidase and Phosphate-Independent Glutaminase of Rat Kidney Cortex. *Can. Fed. Biol. Soc.* 18, 149 (1975).
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